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Purification and Characterization of Pituitary Bovine Somatotropin*

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Bovine somatotropin (bST) has been isolated from pituitary glands and compared in a variety of chemical analyses and bioassays with somatotropin derived from recombinant *Escherichia coli*. Comparison of pituitary extracts and purified bST by Western blot analysis of two-dimensional gels suggested that the immunoreactive somatotropin species present in the extract were also present in the purified material, with no significant losses or degradation as a result of the purification method. NH_2-terminal sequence analysis indicated the presence of equal quantities of Ala-Phe-Pro-Ala-Met-Ser-Leu-Ser- and Phe-Pro-Ala-Met-Ser-Leu-Ser- sequences. The Met-Ser-Leu-Ser-NH_2-terminal sequence, a degradation product observed in NIH standard lots, was not detected. Assay of bioactivity in a bovine liver receptor-binding assay and in a female rat growth assay showed pituitary bST and recombinant methionyl-bovine somatotropin to be equipotent. Tryptic maps and sequence analysis of pituitary-derived somatotropin suggest the presence of isoaspartate derivatization at Asp^{128}.

Bovine somatotropin has been isolated from pituitary glands by a variety of methods, resulting in preparations with varying degrees of heterogeneity (1). Deletion of NH_2-terminal residues has been observed in somatotropin prepared from acidic extracts and appears to be the result of proteolysis during isolation (2). However, the amino-terminal sequence of highly purified pituitary bovine somatotropin has been shown to contain only Ala-Phe-Pro-Ala-Met-Ser-Leu-Ser- and Phe-Pro-Ala-Met-Ser-Leu-Ser- in approximately equimolar amounts, which appear to result from signal peptide processing during secretion (3). Other observed heterogeneity includes an allelic variation at position 126, which leads to a 2:1 ratio of leucine to valine in preparations from pooled glands (4). In addition, bovine somatotropin fractions from anion-exchange chromatography have been shown to have different biological activities in a panel of bioassays (5). Thus, bovine somatotropin appears as a mixture of related polypeptides that may differ in function or in site of action.

To characterize pituitary somatotropin better, we describe here a purification method that allows isolation of highly purified protein in gram quantities. The methodology employed resulted in a 2–4-fold greater yield of somatotropin than reported previously (1), while maintaining excellent purity. Extensive biochemical analysis of the pituitary-derived material has been carried out to identify microheterogeneity undetected previously. In order to assess the effects of such microheterogeneity on activity, bovine somatotropin from recombinant *Escherichia coli* has been compared with pituitary somatotropin in several biological assays.

EXPERIMENTAL PROCEDURES

Pituitary bST[1] *Purification*—Bovine pituitaries were obtained within 1 h of slaughter, placed on ice for 2–4 h, then stored frozen at −80 °C. All subsequent purification steps were carried out at 0–4 °C. The initial extraction of 33 whole pituitary glands (66 g, wet weight) in 150 ml of extraction buffer was at low speed for 10 min using a Waring blender controlled by a rheostat. Extraction buffer was 4.5 M urea, 50 mM Tris, pH 8.8, 1 mM phenylmethylsulfonyl fluoride 1 mM benzamidine HCl, and 4 mM EDTA. The resulting suspension was further homogenized for 10 min using an Ultra-turrax (Tekmar Co.). This extract was subjected to centrifugation for 15 min at 9,000 rpm in a Sorvall GS-3 rotor. The resulting clear red supernatant was removed by pipette from beneath a white floating layer of lipid-like material. The white layer was discarded, the pellet was extracted as before for 10 min using the Ultra-turrax, supernatant was collected as above, and pooled with the first supernatant.

This extract was adjusted to a pH of 10.2, deionized, and subjected to centrifugation for 20 min in a Beckman Ti-45 rotor at 25,000 rpm to remove turbidity. The solution was loaded onto a 10-cm diameter × 12-cm DEAE-cellulose column (Whatman DE52) which had been equilibrated in 4.5 M urea, 50 mM Tris base. Elution was carried out with a 14-liter gradient composed of equal volumes of equilibration buffer and 4.5 M urea, 50 mM Tris-HCl, pH 8.85, at a flow rate of 0.4 liters/h. Fractions were assayed by reversed-phase HPLC and SDS-PAGE, and those fractions containing bST were pooled and then concentrated using an Amicon YM-10 membrane in a stirred cell apparatus.

Buffer exchange of the pool was performed using a Sephadex G-25 column equilibrated and eluted in 4.5 M urea, 5 mM sodium phosphate, pH 6.9. The G-25 pool was loaded onto a 5-cm diameter × 12-cm carboxymethyl (CM)-cellulose column equilibrated in the G-25 column buffer and eluted with a 2-liter gradient from 5–25 mM sodium phosphate at 0.3 liter/h. Fractions containing bST were pooled and dialyzed *versus* 5 mM sodium bicarbonate, pH 10, and finally dialyzed *versus* water to reduce the sodium bicarbonate concentration to <1% of protein mass. The dialysate was lyophilized and stored at −20 °C.

Reversed-phase HPLC—Reversed-phase HPLC analysis of somatotropin was performed using a 0.4 × 10-cm Econosphere 300 C8 cartridge column with a 0.4 × 1.0-cm C4 guard cartridge (Alltech), using an acetonitrile gradient in 0.1% trifluoroacetic acid. Elution was monitored by UV absorbance at 220 nm. Quantitation of bST samples was by comparison of the bST peak area with a standard curve prepared using methionyl-bST.

SDS-PAGE—Gel electrophoretic analysis for routine screening of column fractions was performed using the Pharmacia LKB Biotech-

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[1] The abbreviations used are: bST, bovine somatotropin; HPLC, high pressure liquid chromatography; RP, reversed-phase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

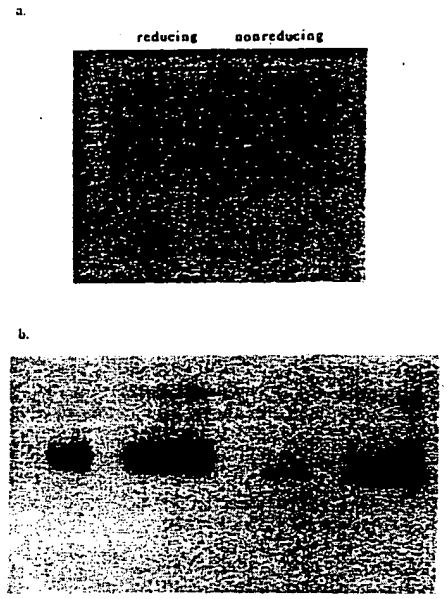

FIG. 2. **SDS-PAGE and Western blot analysis of pituitary bST.** *a*, SDS gels were 15% acrylamide, 0.75 mm thick, and were run for 4 h at 15 mA, constant current. Samples in *lanes 1–7* were reduced prior to loading, and *lanes 9–15* are the same samples under nonreducing conditions. *Lane 1*, M_r markers (BRL prestained); *lane 2*, methionyl-bST; *lane 3*, pituitary bST, CM-cellulose pool; *lanes 4–7*, RP-HPLC (Fig. 1) peaks 1–4, respectively. *Lane 8*, blank. *b*, Western blot of above gel probed with anti-bST antibody. The gel was electrotransferred to APT paper (Schleicher & Schuell) which was probed with antibody, stained with ^{125}I-protein A (Amersham Corp.), and exposed to film for 4.5 h at −80 °C with an intensifying screen.

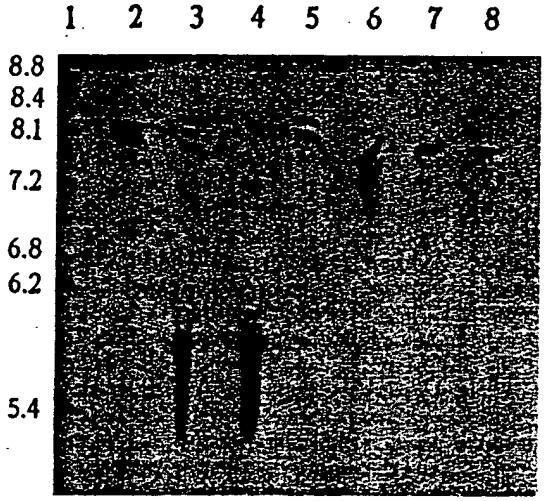

FIG. 3. **Isoelectric focusing analysis of pituitary bST.** Samples (100 μg) were applied to an agarose gel containing pH 5–9 ampholytes and electrophoresed. Solid samples were solubilized in urea/Tris buffer, pH 9.0, at 4 °C and then applied to the gel. *Lane 1*, IEF standards (Pharmacia); *lane 2*, Parlow pituitary bST; *lane 3*, pituitary extract; *lane 4*, DEAE-cellulose load; *lane 5*, CM-cellulose load; *lane 6*, CM-cellulose pool; *lane 7*, methionyl-bST, lot A; *lane 8*, methionyl-bST, lot B.

dominantly bST and also that trace amounts of immunoreactive bST species of apparent M_r greater and less than 22,000 were generated by the RP-HPLC isolation conditions.

Chemical Analysis of Pituitary bST—Analysis by IEF gels was carried out as an assay for charge heterogeneity. Fig. 3 shows the IEF pattern of pituitary somatotropin at various stages of purification and also compares pituitary bST, recombinant methionyl-bovine somatotropin, and NIH standard bST received from A. Parlow (Harbor-UCLA Medical Center). In agreement with previous reports (1, 13), pituitary bST prepared by the method described in this report contained only two IEF bands, which correspond to the alanine (pI = 8.0) and phenylalanine (pI = 7.8) NH_2-terminal species described below. Purified recombinant somatotropin contained minor amounts of at least three isoforms, whereas the NIH standard bST contained numerous bands in addition to the two bands corresponding to bST.

To ascertain whether our preparation of bST contained all of the immunoreactive bST components present in the original pituitary extract, two-dimensional gel electrophoretic analysis was performed as described under "Experimental Procedures." The electrophoretic pattern for the pituitary extract was compared with the purified product by silver staining and by Western blot analysis using an anti-bST antibody probe (Fig. 4, *a–d*). The silver-stained gels were used to compare the relative abundance of somatotropin species, and Western blot analysis was used to identify qualitatively immunoreactive bST species present.

Comparison of Fig. 4, *a–d*, has clearly identified bST as a basic protein of M_r 22,000 which is abundant in the pituitary extract (marked by an *arrow*). Direct comparison of the pituitary extract and purified bST gels indicated that no significant loss of immunoreactive bST occurred during purification. The Western blot data in these gels show two or three acidic immunoreactive bST species in the extract which are not present in the purified material (Fig. 4, *c* and *d*). However, the relative amount of these components is less than 5% of the total mass of bST, as judged by silver-staining intensity. Therefore, the goal of obtaining a purified bST sample that was representative of the material present in the pituitary extract has been attained.

Two-dimensional gel analysis was also performed on the NIH standard pituitary bST (from A. Parlow) as shown in Fig. 4, *e–f*. The Western blot of this gel (Fig. 4*f*) displayed trace amounts of several immunoreactive species of M_r lower than 22,000, indicating the presence of proteolytic cleavages in the protein. The silver-stained gel, however, indicated that the des(1–4) bST proteolysis product was the major contaminant by mass, with the other species present only in trace amounts. From the relative intensities of the two *spots* in Fig. 4*e*, the des(1–4) bST (*lower band*) was equimolar with full length bST, a result also supported by NH_2-terminal sequencing of the mixture (Table II).

NH_2-terminal amino acid sequence of the pituitary bST CM-cellulose pool material revealed two sequences: Ala-Phe-Pro-Ala-Met-Leu-Ser-Leu-Ser- (36%) and Phe-Pro-Ala-Met-Ser-Leu-Ser- (64%). This heterogeneity at the NH_2 terminus of bST has been shown to result from proteolysis at successive peptide bonds during removal of the signal peptide of the somatotropin precursor protein during secretion (3). No other sequences were detectable. In particular, the Met-Ser-Leu-Ser- and Ser-Leu-Ser-Gly-Leu- species detected in pituitary bST samples purified by other methods (14) were not present in the pituitary bST preparation of this study.

Tryptic mapping by reversed-phase HPLC has proven to be an analytical tool with high resolution for amino acid sequence heterogeneity in proteins (15). Pituitary somatotropin was digested with trypsin, and the resulting digest was analyzed by RP-HPLC. Individual peaks were isolated and identified by amino acid composition or by NH_2-terminal sequencing, as necessary. Fig. 5 contains the tryptic map of bST as well as a map of the Ala^{-1}-Val^{126} variant of somato-

of the position 126 allelic variation upon the spectral properties of the protein has not yet been determined.

Due to the extensive homology between prolactin and somatotropin (18) and the possibility of copurification of the two proteins, it was desirable to perform an independent assay of the purified pituitary bST for contaminating prolactin. Reversed-phase HPLC analysis was performed, and no peak was detected in the bST chromatogram at the retention time expected for prolactin (chromatogram not shown), even at column loadings that would detect prolactin at the 1% level. In a Western blot assay of pituitary bST for prolactin using an antibody raised against highly purified prolactin, a small but distinct signal was observed for prolactin. However, the presence of trace amounts of somatotropin in the prolactin used for antibody production cannot be ruled out. The possibility of cross-reactivity of the anti-prolactin antibody for bST in this assay also exists. Collectively, the HPLC and Western blot assays have shown the pituitary bST preparation to be essentially free of prolactin.

Phosphorylation *in vivo* has been postulated as a mechanism for attenuation of activity of somatotropin and other pituitary hormones (19). Therefore, analysis of phosphorylation in pituitary bST has been performed using solid-state ^{31}P NMR. In contrast to a previous report of the presence of phosphate esters in ovine somatotropin (20) from an NIH standard lot, no detectable phosphorous was found in pituitary bST prepared by the method described here, as shown in Fig. 6. Based on a phosphoserine standard, we estimate that there could be no more than 4 μg of phosphoserine in the 175-mg pituitary bST sample that was examined.

Biological Activity of Pituitary bST—Having established that the pituitary bST preparation contained both the expected amino acid sequence variants at the NH_2 terminus and at position 126 as well as a postulated isoaspartate derivative at position 128, the effect of these variations upon biological activity was investigated. Fig. 7 shows the results of a bovine liver receptor-binding assay of pituitary bST and methionyl-bST. Both proteins showed identical binding affinity for bovine liver receptors in the assay. None of the sequence or

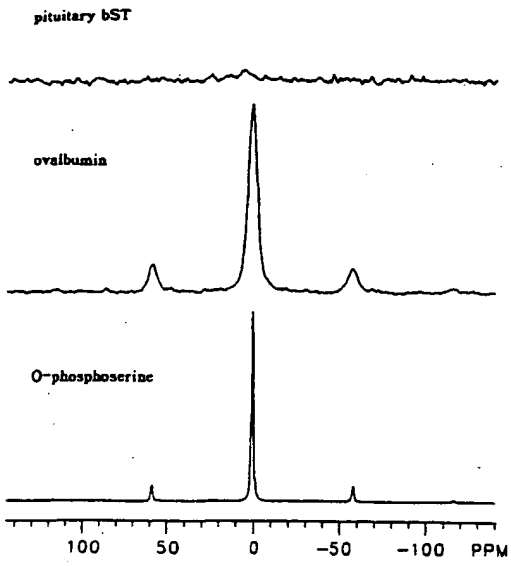

FIG. 6. **Cross-polarization magic angle spinning ^{31}P NMR spectra of phosphorylated amino acids and proteins, V_r = 3 kHz.** *Bottom*, *O*-phosphoserine; *middle*, ovalbumin, sample weight = 273 mg, 60,000 transients; *top*, bovine somatotropin isolated from pituitary glands, sample weight = 175 mg, 60,000 transients.

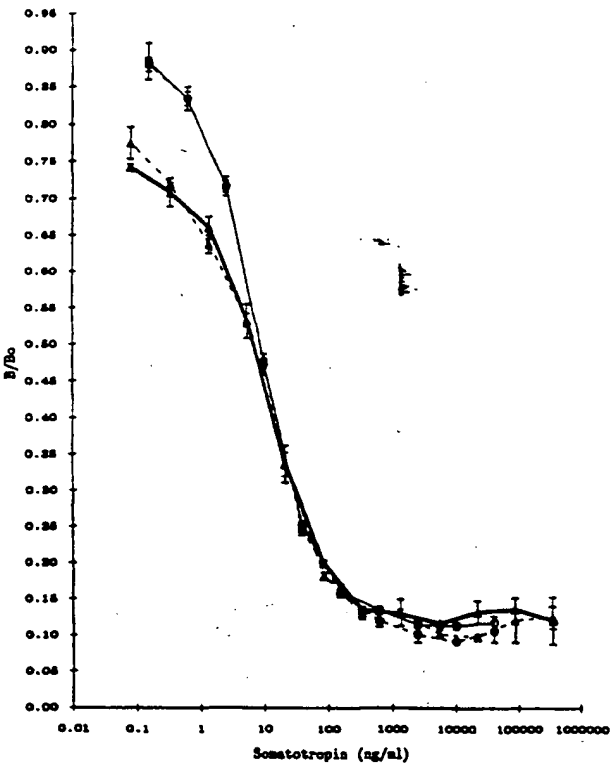

FIG. 7. **Radioreceptor assay for bovine somatotropin.** Bovine liver somatotropin radioreceptor assays were carried out as described under "Experimental Procedures." Results are shown as $(B-B_{nsb})/(B_0-B_{nsb})$ (where B = counts bound, B_0 = counts bound in the absence of competitor, and B_{nsb} = counts bound in the absence of receptors) for increasing concentrations of unlabeled competitor. The *error bars* represent the 95% confidence range determined for each set of three replicates. The competition of a standard recombinant bovine somatotropin preparation (●) and (○) and a preparation of pituitary bovine somatotropin (▲) and (△) with ^{125}I-rbST for bovine liver receptors is shown. *Open* and *closed symbols* represent separate experiments.

structural differences between the pituitary and recombinant preparations had an apparent effect upon this measure of somatotropin activity.

The protein preparations were also compared in an *in vivo* assay in which weight gain in mature female rats was determined. As shown in Fig. 8, no statistically significant differences in potency were observed between methionyl-bST and pituitary-derived somatotropin.

Previous studies of the galactopoietic response to somatotropin in dairy cattle (21) have demonstrated that NIH standard lots of bST obtained from A. Parlow displayed lower milk production activity than any of several recombinant somatotropins, including the Met^{-1}-Leu^{126}, Met^{-1}-Val^{126}, Ala^{-1}-Leu^{126} bST, and Ala^{-1}-Val^{126} bST variants. However, the Parlow bST preparations were subsequently shown by NH_2-terminal sequence analysis to contain 40–50% des(1–4) molecules, as shown in Table II. In a subsequent study (22), recombinant des(1–4) somatotropin (NH_2-terminal sequence 20% Met-Ser-Leu-Ser—, 80% Ser-Leu-Ser) was purified, assayed for galactopoietic activity, and also found to have decreased activity relative to full length recombinant bST.

DISCUSSION

A purification procedure for bovine somatotropin from pituitaries has been developed which results in protein of high

should not occur at an appreciable rate under the conditions used in this paper for isolation of bST from pituitary (16). In studies of carboxylmethyltransferase activity using glucagon as a substrate, Ota *et al.* (24) determined that base treatment of glucagon (0.1 M NH_4OH, pH 10.1, 37 °C, 3 h) resulted in rearrangement to L-isoaspartate at some asparagine residues but not at aspartate residues. Therefore, the conditions of 50 mM Tris base, pH 10.3, 4 °C used in the DEAE-cellulose chromatography step in this preparation of pituitary bST appear unlikely to have yielded the observed level of isoaspartate.

These data suggest that the Asp^{128} β-linked bST protein may be a component of somatotropin *in vivo*. The rate of isoaspartate formation *in vivo* has not been measured directly, but model peptide studies of rearrangement at an Asp-Gly sequence (Asp^{128} in bST is also followed by glycine) suggest that the rate is slow at physiological temperature and pH, with a $t_{\frac{1}{2}}$ on the order of weeks (16). However, the rate of reaction in proteins at a given aspartate residue is probably a function of tertiary structure as well as sequence (24, 25). Carboxylmethyltransferase activity, which has been proposed to be involved in repair of isoaspartyl damage in proteins by restoring the peptide bond (26), may have a role in determining the observed level of isoaspartate in bovine somatotropin. This activity has been isolated from the cytosolic fraction of pituitary tissue, although it is not yet known whether the enzyme is present in secretory granules harboring bST (27). The observed level of Asp^{128} derivatization in pituitary bST may prove to be a complicated function of the rates of isomerization, carboxylmethyltransferase activity, and secretion.

Our analysis of pituitary bST for phosphorylated amino acids using ^{31}P NMR has demonstrated conclusively that the preparation contains no significant levels of phosphorus. This result is in contrast to a previous report that phosphate esters were present in pituitary ovine somatotropin (20). The mild conditions used for isolation in the method described herein have eliminated the possibility that phosphorus was lost during purification. The possibility exists that phosphorylation is specific to the ovine system and is not present in bovine, although this seems unlikely. More probably, the presence of phosphorylation in the ovine preparation was due to nonspecific kinase activity encountered during purification, which was avoided by the method described in this report.

To assess the effect of pituitary bST microheterogeneity on activity, both direct binding studies to receptors in bovine liver membranes and growth response in a rat growth model were employed. For both assays, pituitary bST and recombinant somatotropin were found to have equivalent specific activities, as shown in Figs. 7 and 8. Similar conclusions regarding biological activity have been reached by other investigators comparing recombinant bovine somatotropin with pituitary bST obtained from NIH through the National Hormone and Pituitary Program (U. S. A.) (28).

In addition to demonstrating the equal potency of recombinant and pituitary bST, the activity assay results have suggested that the proposed β-linked component at Asp^{128} contains substantial activity. If this component comprising 20% of the protein mass were totally inactive, the decrease would have been detectable by the rat growth assay.

The rat growth assay employed has been shown to correlate with decreased galactopoietic activity in dairy cows (22) and therefore appears to be a valid predictor of milk yield for bST variants less active than methionyl-bST. Although the pituitary bST preparation described here has not yet been assayed directly for galactopoietic activity, we postulate that full activity would be observed. The decreased activity of the NIH standard lot of pituitary bST obtained from A. Parlow relative to recombinant methionyl-bST in the earlier studies (21) was due most likely to the high level of NH_2-terminal deletion variants present in the Parlow preparation (see Table II).

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APPENDIX A

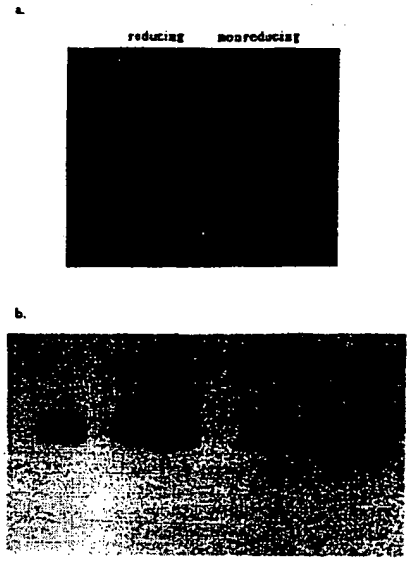

FIG. 2. SDS-PAGE and Western blot analysis of pituitary bST. *a*, SDS gels were 15% acrylamide, 0.75 mm thick, and were run for 4 h at 15 mA, constant current. Samples in *lanes 1–7* were reduced prior to loading, and *lanes 9–15* are the same samples under nonreducing conditions. *Lane 1*, M_r markers (BRL prestained); *lane 2*, methionyl-bST; *lane 3*, pituitary bST, CM-cellulose pool; *lanes 4–7*, RP-HPLC (Fig. 1) peaks 1–4, respectively. *Lane 8*, blank. *b*, Western blot of above gel probed with anti-bST antibody. The gel was electrotransferred to APT paper (Schleicher & Schuell) which was probed with antibody, stained with ^{125}I-protein A (Amersham Corp.), and exposed to film for 1.5 h at −80 °C with an intensifying screen.

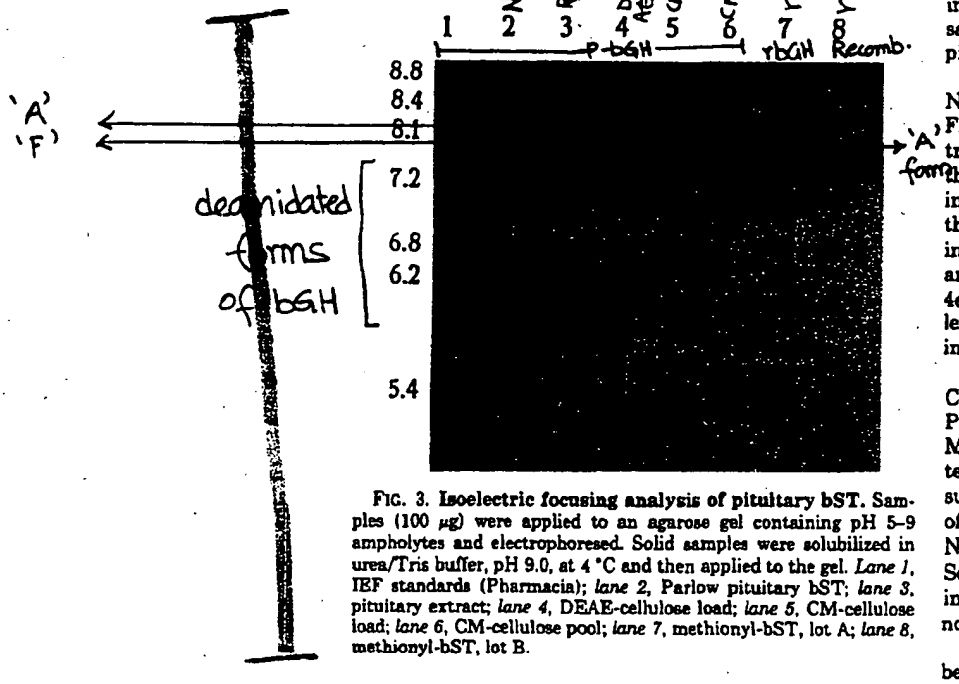

FIG. 3. Isoelectric focusing analysis of pituitary bST. Samples (100 μg) were applied to an agarose gel containing pH 5–9 ampholytes and electrophoresed. Solid samples were solubilized in urea/Tris buffer, pH 9.0, at 4 °C and then applied to the gel. *Lane 1*, IEF standards (Pharmacia); *lane 2*, Parlow pituitary bST; *lane 3*, pituitary extract; *lane 4*, DEAE-cellulose load; *lane 5*, CM-cellulose load; *lane 6*, CM-cellulose pool; *lane 7*, methionyl-bST, lot A; *lane 8*, methionyl-bST, lot B.

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Chemical Analysis of Pituitary bST—Analysis by IEF gels was carried out as an assay for charge heterogeneity. Fig. 3 shows the IEF pattern of pituitary somatotropin at various stages of purification binant methionyl-bo bST received from A ter). In agreement wi prepared by the met only two IEF bands, 8.0) and phenylalani scribed below. Purifi minor amounts of at standard bST contai two bands correspon

To ascertain whet of the immunoreacti nal pituitary extrac analysis was perfor Procedures." The el extract was compar staining and by We antibody probe (Fig. to compare the relat and Western blot an immunoreactive bST

Comparison of Fig basic protein of M_r 2 extract (marked by pituitary extract an significant loss of im rification. The West three acidic immunor are not present in t However, the relativ than 5% of the total r intensity. Therefore, sample that was repr pituitary extract has

Two-dimensional g NIH standard pituit Fig. 4, *e–f*. The Wes trace amounts of seve than 22,000, indicati in the protein. The si the des(1–4) bST pro inant by mass, with amounts. From the re 4*e*, the des(1–4) bST length bST, a result a ing of the mixture (T

NH_2-terminal amin CM-cellulose pool ma Pro-Ala-Met-Leu-Ser Met-Ser-Leu-Ser- (6 terminus of bST has successive peptide bo of the somatotropin No other sequences Ser-Leu-Ser- and S in pituitary bST samp not present in the pit

Tryptic mapping b be an analytical tool sequence heterogeneit pin was digested wit analyzed by RP-HPL identified by amino sequencing, as necess bST as well as a map

APPENDIX - B

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TABLE I

and growth hormone preparations

Reference	Solubilization procedure	Average yield
		mg/g whole pituitary
12	75% Ethanol, 150 mM NaOH, 3 h, 4 °C[a]	0.8
13	75% Ethanol, pH 10.5, 10 h, 4 °C[a]	1.5
14	1% Deoxycholate, pH 8.4, 20 h, 4 °C	2.0
This work	1 mM EGTA, 10 mM NH_4HCO_3, pH 8.0, 40 min, 37 °C	0.7
3	$Ca(OH)_2$, pH 11.5, 24 h, 4 °C	1.6[b]
11	$Ca(OH)_2$, pH 10.5, 1 h, 4 °C, freeze-thaw	2.0
15	$Ca(OH)_2$, pH 11.5, 24 h, 4 °C	0.6[b]
14	0.1 M Sodium phosphate, pH 5.5, 16 h, 4 °C, twice	4.0
16	Freeze-thaw, 25 mM NaCl, 6.5 mM $Na_2B_4O_7$, pH 8.7, 3 h, 0 °C	1.9[b]
13	250 mM $(NH_4)_2SO_4$, pH 5.5, 16 h, 4 °C[a]	1.0
17	250 mM $(NH_4)_2SO_4$, pH 5.5, overnight, 4 °C[a]	1.1
This work	1 mM EGTA, 10 mM NH_4HCO_3, pH 8.0, 40 min, 37 °C	1.0

ter previous extractions according to Ellis (18).
if weight of anterior pituitary tissue used. A conversion factor of
ae applied.

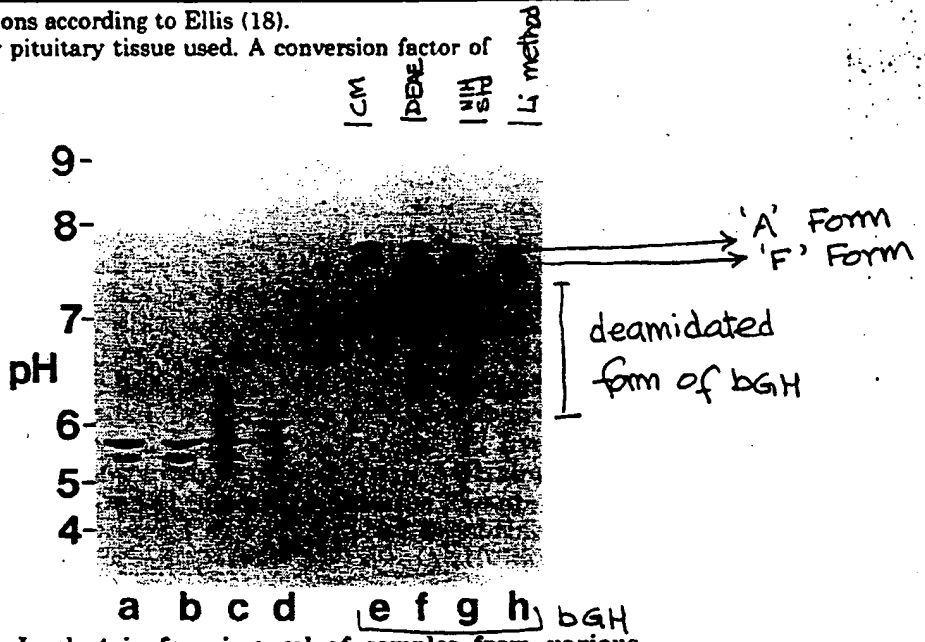


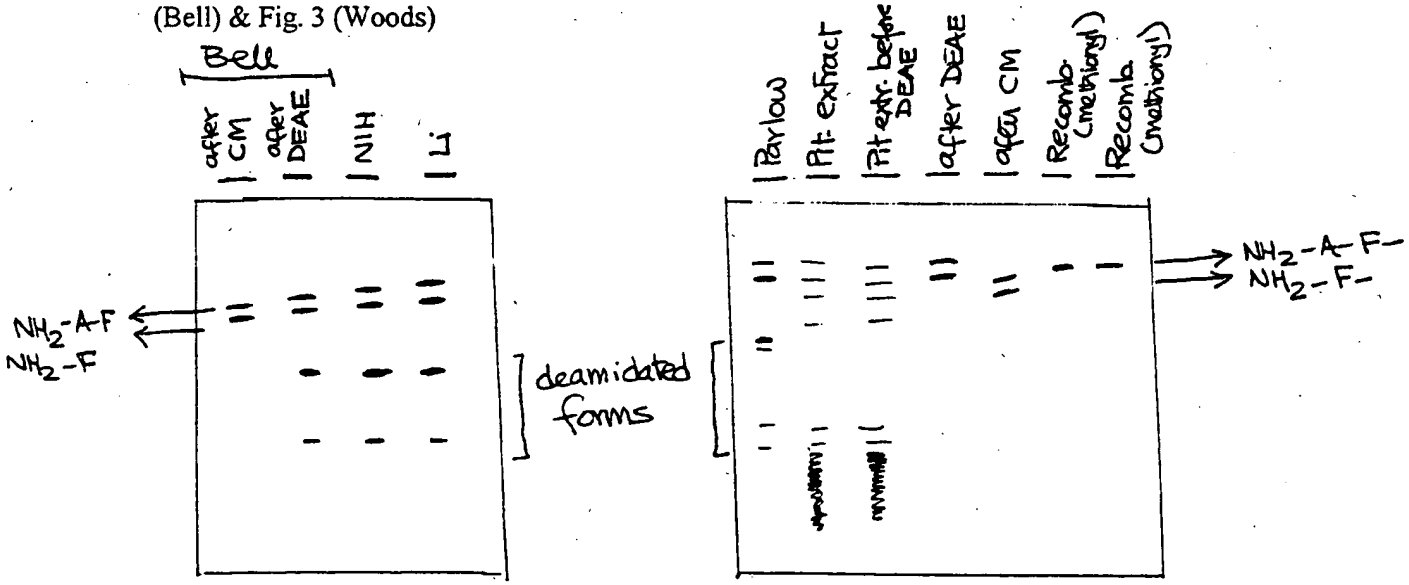
FIG. 1. **Isoelectric focusing gel of samples from various growth hormone and prolactin preparations.** The samples analyzed were: *lane a*, bovine prolactin after preparative electrophoresis; *lane b*, bovine prolactin after DEAE-cellulose column; *lane c*, bovine prolactin obtained from the National Institutes of Arthritis, Diabetes, and Digestive and Kidney Disease, Lot NIH-P-B4 (13); *lane d*, ovine prolactin, gift from Dr. C. H. Li (29); *lane e*, bovine growth hormone after CM-cellulose column; *lane f*, bovine growth hormone after DEAE-cellulose column; *lane g*, bovine growth hormone obtained from Lot NIH-GH-B18 (13); *lane h*, bovine growth hormone, gift from Dr. C. H. Li (11). Each lane contained a total of 10 μg of protein. The approximate pH at the edge of the gel is shown.

previously observed with human growth hormone (21). The

APPENDIX C

Daniel Method (1966)	Reichert Method (NIH sample)	Bell Method (1985)	Wood method (1989)
bGH known in the art in 1966 (Li/ Wilhemi)	Extract with ammonium sulfate & centrifuge	Unlike previous methods, bGH extracted in the absence of organic solvents & extreme pH	bGH extracted from pituitary in urea—different from the Bell method
↓	↓	↓	↓
Gel-exclusion chromatography	Re-extract & centrifuge 3 more times (65% pure)	ion-exchange chromatography (DEAE) (80% pure)	ion-exchange chromatography
↓	This method is based on Ellis method published in 1961.	↓	↓
Pool samples with bGH & dialyze (dialysis removes salts)		Pool samples with BST, concentrate, buffer exchange by gel exclusion chromatography	Pool samples with BST, concentrate, buffer exchange by gel exclusion chromatography
		↓	↓
		Carboxymethyl (CM)-cellulose column	(CM) column
		↓	↓
		Pooled and dialyzed (>95% pure)	Pooled and dialyzed (>98% pure)

Product derived by Li, Reichert, Bell & Wood's DEAE steps look the same on IEF. *See* Fig. 1 (Bell) & Fig. 3 (Woods)



Proteins are separated by CHARGE in IEF.

Exhibit B

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Purification and Physiochemical Properties of a Recombinant Bovine Growth Hormone Produced by Cultured Murine Fibroblasts

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ABSTRACT. Mouse fibroblast cell lines which secrete bovine (b) GH have been generated. This was accomplished by cotransforming mouse L cells (thymidine kinase-negative [TK^-] and adenine phosphoribosyl transferase-negative [$APRT^-$]) with DNA molecules encoding the Rous sarcoma virus-long-terminal repeat and bGH genes along with the herpes viral TK gene and the hamster APRT gene. One stable cell line, Pdλ-pbGH 4-13, was found to secrete approximately 75 μg bGH per 24 h/5.0 × 10^6 cells. Media from this cell line were collected for purification of recombinant bGH (rbGH). Purification involved $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography, and gel filtration on Sephacryl S-200. The rbGH was characterized by bioassay, RIA, radioreceptor assay, and sodium dodecyl sulfate gel electrophoresis. Results of these analyses were compared with those obtained with a highly purified pituitary bGH. In the rat tibia bioassay, rbGH was found to be as potent as pituitary bGH. Results from the RIA, radioreceptor assay, and sodium dodecyl sulfate gel electrophoresis and Western blot analysis also suggested that the rbGH was similar to that of pituitary origin. Amino acid composition, partial (amino-terminal) sequence, and tryptic peptide maps were also found to be similar between the rbGH and pituitary bGH preparations. The amino terminus of the rbGH showed similar heterogeneity to that of the bGH of pituitary origin. We conclude that rbGH which was synthesized, processed, and secreted from transformed mouse fibroblasts possessed almost exactly the same physiochemical properties as pituitary bGH. (*Endocrinology* **119**: 1489–1496, 1986)

BOVINE GH (bGH), a 22,000-mol wt protein, was the first GH isolated and characterized from pituitary glands (1). GH, which is synthesized in and secreted by the anterior pituitary lobe, is necessary for normal growth and development. In classic experiments, it was shown that hypophysectomy stunts growth in animals, and exogenous administration of pituitary extract or purified pituitary GH reverses these effects (2, 3). GH not only stimulates proportional growth in animals but also influences protein, carbohydrate, and rat metabolism (4, 5). Hayashida *et al.* (6) showed, by immunochemical techniques, that during evolution the GH molecules retained a high degree of structural conservation. Indeed, most of the GHs which have been sequenced from different species show a high degree of homology (7). An example of species differences in the ability to respond to the different GH is that human dwarfs respond to primate GH but not to subprimate GH preparations (4, 8). GH from all mammalian species promotes growth in the hypophysectomized rat and is active in the rat tibia bioassay [(BA) Ref. 4].

GH isolated from pituitary glands is heterogeneous (9–11). A 20,000-mol wt variant of human GH (hGH), isolated and characterized by Lewis *et al.* (12), is formed by the deletion of residue 32–46 (13). Such deletions could be caused by the alternate processing of the hGH-messenger RNA (mRNA) precursors, as reported by DeNoto *et al.* (14). The 20 K variant hGH has different biological, receptor-binding, and immunoactivities from those of the 22 K major form of hGH (10). The 20,000-mol wt variant of bGH has not yet been isolated. Thus, the question remains as to whether different forms of GH in the bovine species have different biological activity. Hart *et al.* (15) have reported an isohormone of bGH which possesses more growth activity than immunoactivity, although it has no diabetogenic activity.

With recombinant DNA technology, GH genes have been expressed in both prokaryotic and eukaryotic cells (16–24). However, only hGH and bGH have been purified to homogeneity from their prokaryotic expression in *Escherichia coli*. They differ from the pituitary form of GH in having an extra methionine at the amino terminus as a result of the adenosine-uridine-guanosine start codon inserted at the beginning of the gene (16–18, 25).

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We have used an avian retroviral long-terminal repeat (LTR) to direct expression of a genomic plasmid clone of bGH to successfully establish a stable cell line which secretes bGH (26). This paper describes the isolation and purification, and the physiochemical and biological properties, of the recombinant bGH (rbGH).

Materials and Methods

Cell culture and purification

Mouse L cells [thymdine kinase-negative (TK^-) and adenine phosphoribosyl transferase-negative ($APRT^-$)] were cotransformed with DNA molecules encoding the Rous sarcoma virus (RSV-LTR) and bGH genes along with the herpes viral TK gene and the hamster APRT gene. A stable, bGH-secreting mouse cell line, L-Pdλ-bGH4-13, was expanded into roller bottles and maintained in Dulbecco's modified Eagle's medium, 10% calf serum, 15 μg/ml hypoxanthine, 1 μg/ml aminopterin, and 15 μg/ml thymidine. The medium was collected at 3- to 4-day intervals and pooled. Pooled medium was frozen at −20 C until purification.

Approximately 1 liter of pooled serum was thawed and saturated to 50% with $(NH_4)_2SO_4$. The precipitate which formed was dialyzed and lyophilized, and it was further fractionated on diethylaminoethyl cellulose column equilibrated with 0.3 M Tris-HCl, pH 7.4, buffer. Most of the GH, which was not absorbed onto the column, was in the flow-through fractions. The GH-rich fractions were then passed through a Sephadex G-25 column (2.5 × 100 cm) equilibrated with 0.05 M NH_4HCO_3, then further purified in the same buffer (0.05 M NH_4HCO_3) on a Sephacryl S-200 column (2.5 × 100 cm). The purification was followed with a homologous RIA for bGH. A yield of approximately 30% was routinely obtained from the starting material (10–15 mg/liter bGH).

BA, RIA and radioreceptor assay (RRA)

A highly purified pituitary bGH, obtained from Dr. C. H. Li (University of California, San Francisco, CA), was iodinated by a modification of the lactoperoxidase procedure of Thorell and Johansson (27), previously described (28). The free iodine was separated from the protein-bound iodine by chromatography on a Sephacryl S-200 column equilibrated with 0.05 M PBS containing 1% BSA, and the labeled hormone was diluted in PBS-BSA buffer to a working concentration of 10,000 cpm/100 μl.

We generated and characterized antibody to purified pituitary bGH for the development of a homologous bGH-RIA in the following manner. Purified pituitary bGH was dissolved in 1 ml sterile saline at 100 μg/ml and emulsified with an equal volume of Freund's complete adjuvant. Rabbits were immunized by procedures described by Vaitukaitis *et al.* (29). Booster injections were given at 4- to 6-week intervals; they involved use of 50 μg bGH per rabbit and incomplete Freund's adjuvant. Blood was collected from ear veins and screened with [^{125}I]bGH for titer determination. Antibody for bGH (pool 1; AB_1) was used in the homologous RIA at 1:1000 dilution. The specificity of the AB_1 is shown in Fig. 1. bGH and ovine GH (oGH) competed with the [^{125}I]bGH label, but hGH, rat GH (rGH) and chicken GH (cGH) did not. Partial cross-reaction with ovine PRL (oPRL) was observed, possibly because of oGH contamination in the oPRL preparation. Other pituitary hormones [ovine LH (oLH) and ovine FSH (oFSH)] did not compete in the assay, and serum from a hypophysectomized ewe showed no cross-reactivity.

bGH was assayed by standard, double-antibody RIA procedures (28). Results are expressed as either nanograms per ml or micrograms per ml, in terms of reference preparations of bGH (NH-B18) obtained from the National Pituitary Agency, NIADDK.

The cGH RRAs were performed according to procedures previously described (28). In brief, cGH (FLcGH-2), iodinated as described above, was used as the radioligand; 600 μg membrane protein from chicken liver were used as the receptor source in the RRA. The incubation was carried out at room temperature for 16–20 h before the mixture was centrifuged at 4 C. The supernatant was decanted, and bound [^{125}I]cGH was counted in an automatic γ-counter (Micromedic model 10/600; Micromedic Systems, Inc., Horsham, PA). Results are expressed as either nanograms per ml or micrograms per ml, in terms of reference preparations of bGH (NIH-B18) obtained from the National Pituitary Agency, NIADDK.

The biological activity of the GH preparations was assayed in the hypophysectomized rat tibia BA (3).

Chemical and physiochemical characterization

The purity of the bGH was examined by disc gel electrophoresis at pH 8.3 in 7.5% acrylamide gels stained with amido black (30), and by slab gels, according to the methods of Laemmli (31), with sodium dodecyl sulfate (SDS) denaturing condition. The slab gels were run at either 12 or 15% acrylamide, stained with Coomassie brilliant blue R-250, and destained with diffusion in 5% methanol and 7.5% acetic acid.

Electroblotting was performed immediately after the completion of electrophoresis. Proteins from the 15% slab gels were transferred onto nitrocellulose paper as described by Towbin *et al.* (32), with the use of electroblotting equipment (Hoeffer Scientific Instruments, San Francisco, CA). The electroblotting was performed overnight, and the paper was air dried after transfer. The nitrocellulose paper was immunostained with anti-bGH serum (pool 1) at a 1:1000 dilution, according to the procedures of Adair *et al.* (33). The unbound antibody was washed off, and immunostained paper was allowed to react with [^{125}I]Protein A (New England Nuclear, Boston, MA). The reactive bands were demonstrated by autoradiography.

Amino acid composition and sequence determinations

Both the native and rbGH were reduced and carboxymethylated before chemical characterization, tryptic mapping, and partial microsequence analysis (34). This was accomplished by dissolving the protein (5 nmol) in 500 μl 0.5 M ammonium acetate (pH 8) and 6 M guanidine hydrochloride containing 2 μmol dithiothreitol, and incubating the mixture at room temperature for 1 h. After reductive cleavage of the disulfide bonds sodium monoiodoacetate (4 μmol) was added, and the resulting

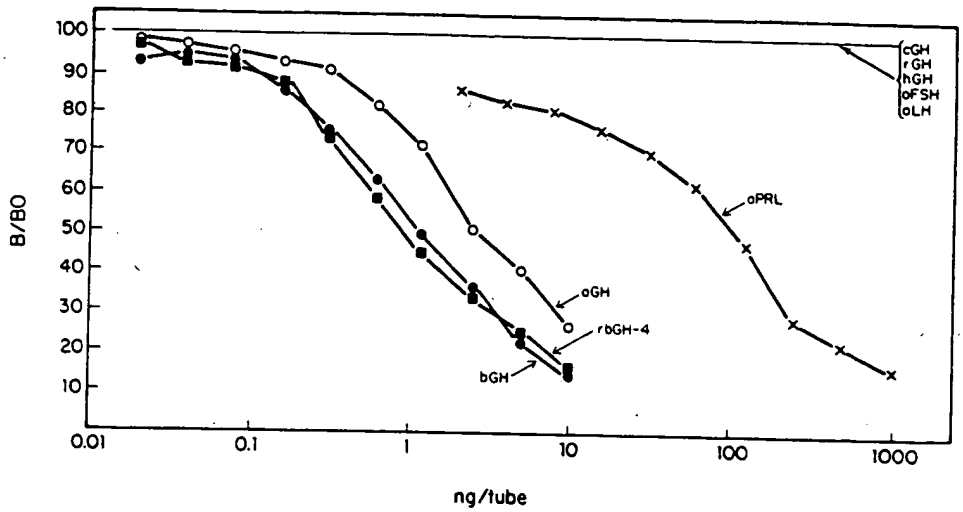

FIG. 1. Displacement curves for bGH (■), rbGH (●); oGH (O); oPRL (×); cGH, rGH, hGH, oFSH, and oLH in a homologous bGH RIA. Each *point* represents the mean of duplicates.

solution was incubated in the dark for 2 h. The reaction was terminated by addition of 2-mercaptoethanol (10 μl), the pH was decreased to 2.5 with trifluoroacetic acid, and the mixture was applied to a Vydac (Rainion Instrument Co., Inc., Woburn, MA) C4 reverse-phase HPLC column (5 μm packing; 33 nm pore size) that had been equilibrated with 0.1% aqueous trifluoroacetic acid. The column was then washed with 20 ml of the same buffer to remove excess reagents, buffer salts, and denaturing agent. The carboxymethylated protein was removed from the HPLC column by eluting with 60% acetonitrile containing 0.1% trifluoroacetic acid. The column effluent was onitored by a fluorescamein detection system (35).

Tryptic digestion of the carboxymethylated proteins and isolation of the tryptic peptides by HPLC on a Vydac C18 column (5 μm packing; 33 nm pore size) were carried out as previously reported (34, 36). Amino acid analyses were performed by HPLC, with the use of precolumn derivatization with phenylisothiocyanate (37, 38). Microsequence analyses were carried out on less than 500 pmol protein with an Applied Biosystems (Foster City, CA) model 470A protein sequencer similar to that described by Hewick *et al.* (39). Phenylthiodantoin derivatives of amino acids were analyzed by HPLC on a Hypersil C18 column (5 μm packing; 10 nm pore size) with absorbance monitored at 269 nm and 313 nm on a Hewlett-Packard (Corvallis, OR) 1090 chromatograph (40).

Hormone

Native pituitary bGH (5882-76-1) was obtained from Merck Chemical Division (Rahway, NJ) and further purified in a Sephacryl S-200 column (2.5 × 90 cm) equilibrated with 0.05 M NH_4HCO_3. The bGH peak (5882-84) was eluted with a V_e/V_o of 2.0 as measured by bGH RIA, and the fractions were pooled and lyophilized. The GH preparations are referred to hereafter as (5882) for the original and (5882-84) for the repurified preparations. The bGH preparations purified from the cell culture medium will be referred to hereafter as rbGH. Recombinant bGH-2 and rbGH-4 are two different purified preparations. hGH (5704-68-1) was obtained from Merck emical Division and cGH (FLcGH-II) was purified by us eviously (28). oGH (NIADDK-oGH-12), oPRL (NIADDK-oPRL-17), oLH (NIH-S15), oFSH (NIH-S24), and rGH (NIADDK-rGH-RP-1) were obtained from the National Hormone and Pituitary Program (Baltimore, MD).

Results

Chemical and physiochemical characterization

Figure 2 shows a typical gel filtration profile of the rbGH run on the Sephacryl S-200 column. It emerged as a single symmetrical peak. Disc gel electrophoresis of the native pituitary and rbGH revealed similar banding patterns, confirming the heterogeneity of the preparations (Fig. 3). The SDS-polyacrylamide gel electrophoretic (PAGE) analysis in 12.0% and 15% acrylimide gels of the native pituitary and rbGH also revealed a similar pattern among the preparations (Fig. 4 and 5-I). The major component is the 22,000-mol wt species GH and a minor component, a dimer of 44,000 mol wt. All preparations showed similar patterns of minor protein bands, most noticeably the species of GH around 20,000 mol wt. When the proteins from the 15% gel were transferred onto the nitrocellulose paper by electroblotting, three prominent bands appeared; the 22,000, 44,000, and 20,000 mol wt bands. However, minor bands also appeared to show cross-reactivity with the bGH antibody (Fig. 5-II). When the same preparations were subjected to isoelectrofocusing analysis, all four appeared to possess multiforms of isohormone having isolectric point (Pi) values of 8.1, 7.9, and 6.8 (Fig. 6).

The amino acid analysis of the native pituitary (5882-84) and rbGH-2 is shown in Table 1 with the composition of bGh reported by Graf and Li (41) for comparison. It is characterized by having four cysteine residues, a high content of glutamic acid, aspartic acid, and leucine and a low content of histidine and methionine. The N-terminal amino acid sequence analysis revealed that both the native pituitary and the rbGH were heterogeneous

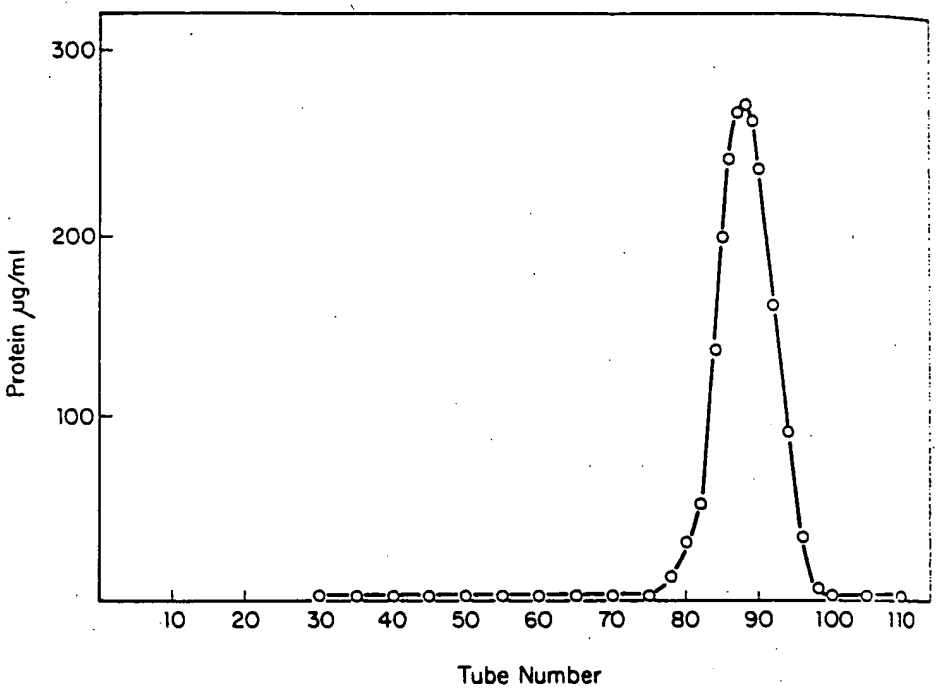

FIG. 2. A typical gel filtration profile of rbGH on a Sephacryl S-200 Column (2.5 × 100 cm) equilibrated with 0.05 M NH_4HCO_3. Protein concentrations were determined by a Bio-Rad (Bio-Rad Laboratories, Richmond, CA) protein dye assay, with BSA as the standard.

FIG. 5. I, SDS-I
tion of mol wt
marker is same
bGH (5882); B,
2; and D, rbGI
of the Western
B, bGH (5882-
4.

FIG. 3. Polyacrylamide disc gel electrophoresis (pH 8.3), amido black stain. Gel was 7.5%. A, bGH (5882), original preparation; B, bGH (5882-84), repurified preparation and two different purified preparations; C, rbGH-2; and D, rbGH-4.

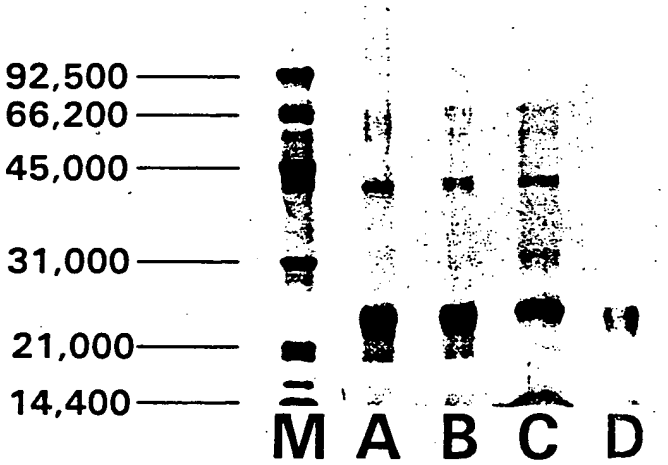

FIG. 4. SDS-PAGE (12%) determination of mol wts of GHs. M, Molecular marker; A, bGH (5882), original preparation; B, bGH (5882-84), repurified preparation and two different purified preparations; C, rbGH-2; and D, rbGH-4.

in their amino-terminal residues. There are aproximately e molar amounts of the native pituitary bGH species, s ing either with alanine or phenylalanine (43%), and minor species starting at position 4 with alanine (14%).

The rbGH contains 51% N-terminal alanine, 30% N-terminal phenylalanine and two minor species, one starting with proline (16%) at position 3 and another at alanine (3%) at position 4 (Table 2). The tryptic mapping of the native (5882-84) and rbGH-2 revealed similar elution profiles (Fig. 7).

BA, RIA, and RRA

In the rat tibia BA (Fig. 8), the response to rbGH was dose dependent and essentially parallel to the responses to bGH 5882 and bGH 5882-84. The rbGH was equipo-

M

FIG. 6. Deter
acrylamide ge
bGH (5882);

tent with
twice as a
The rbGH
the homol
potent tha
the cGH h
rbGH gav
(Fig. 10).

It is ev
purified f
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rbGH bel
GH in th

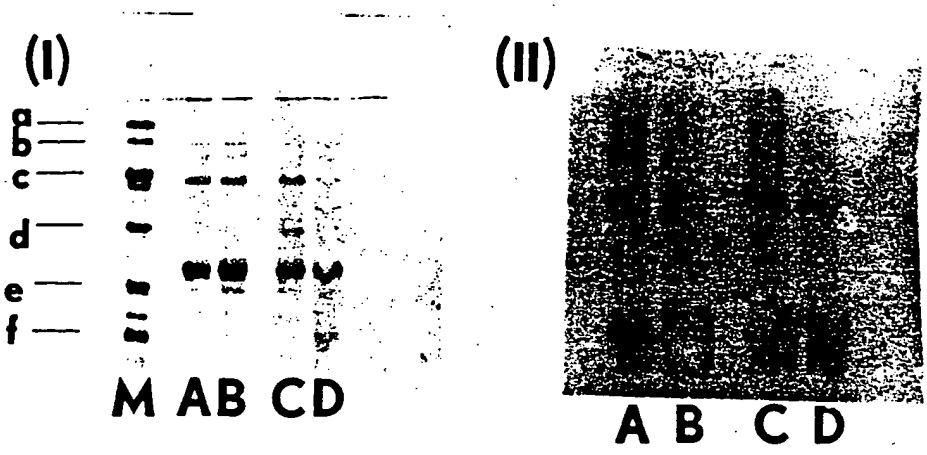

FIG. 5. I, SDS-PAGE (15%) determination of mol wts of GHs. M, Molecular marker is same as described in Fig. 4; A, bGH (5882); B, bGH, 5882-84; C, rbGH-2; and D, rbGH-4. II, Autoradiography of the Western blot of: A, bGH (5882); B, bGH (5882-84); C, rbGH-2; D, rbGH-4.

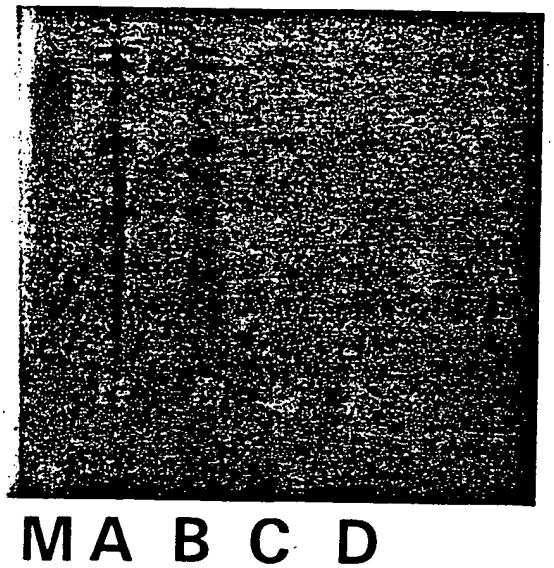

FIG. 6. Determination of the isoelectric point of the bGHs in polyacrylamide gel isoelectric focusing. M, Marker protein, hemoglobin; A, bGH (5882); B, bGH (5882-84); C, rbGH-2; D, rbGH-4.

tent with the purified native bGH (5882-84) and about twice as active as the reference material bGH (5882). The rbGH gave a parallel, dose-dependent response in the homologous RIA, but it seemed to be a little less potent than the two pituitary preparations (Fig. 9). In the cGH hepatic RRA, the pituitary preparation and the rbGH gave indistinguishable dose-displacement curves (Fig. 10).

Discussion

It is evident from the present study that the rbGH purified from a medium of cultured fibroblasts transformed with an avian retroviral-bGH DNA recombinant is similar to that purified from the pituitary gland. The r[illegible]H behaves almost exactly like the native pituitary [illegible]n the rat tibia BA and RIA and in the cGH RRA.

TABLE 1. Amino acid composition of native and rbGH

Amino acid	Native residues		Recombinant residues		Residues from sequence
	bGH	CM-bGH[a]	bGH	CM-bGH	
Asx	14.6	15.6	15.9	15.3	16
Glx	24.0	24.6	24.5	24.5	24
Cmc[b]		4.1		4.2	
Ser	13.6	13.5	13.4	13.6	13
Gly	10.9	10.9	10.8	11.3	10
His	2.9	2.8	3.0	2.8	3
Arg	13.8	13.5	13.6	13.7	13
Thr	12.3	11.9	12.1	11.7	12
Ala	14.9	14.6	15.0	14.7	15
Pro	6.4	6.3	6.5	6.5	6
Tyr	6.0	6.0	5.9	5.9	6
Val	6.6	6.2	6.3	6.1	6
Met	4.1	4.0	4.2	3.8	4
½Cys	3.7		4.0		4
Ile	6.5	6.4	6.2	6.3	7
Leu	26.7	26.3	25.9	26.4	27
Phe	12.5	12.4	12.6	12.5	13
Lys	10.4	10.7	10.3	10.6	11
Trp	ND	ND	ND	ND	1
					191

Samples (100–400 pmol) were hydrolyzed with constant boiling HCl at 150 C for 1 h. The number of residues was calculated assuming 190 residues per molecule. Tryptophan content was not determined (ND).

[a] CM-bGH, Carboxymethylated bGH.

[b] CMC, Carboxymethylcysteine.

The amino acid composition, tryptic mapping, and N-terminal sequence of the rbGH were also shown to be similar to those from bovine pituitary extract of native pituitary origin. Furthermore, the rbGH showed heterogeneity similar to that of native pituitary bGH.

GH is composed of a family of peptides which possesses various biological activities, with well-documented heterogeneity (9–11). In humans, the major physiological species of GH is the 22,000-mol wt polypeptide having 191 amino acid residues. Minor forms are the 20 K variant isolated and characterized by Lewis *et al.* (12)

TABLE 2. Microsequence analysis of native pituitary and rbGH

Peptide	1 2 3 4 5 6 7 8 9 10	% Pituitary bGH	% rbGH
bGH (1-191)	*N*-Ala-Phe-Pro-Ala-Met-Ser-Leu-Ser-Gly-Leu	43	51
bGH (2-191)	*N*-Phe-Pro-Ala-Met-Ser-Leu-Ser-Gly-Leu	43	30
bGH (3-191)	*N*-Pro-Ala-Met-Ser-Leu-Ser-Gly-Leu		16
bGH (4-191)	*N*-Ala-Met-Ser-Leu-Ser-Gly-Leu	14	3

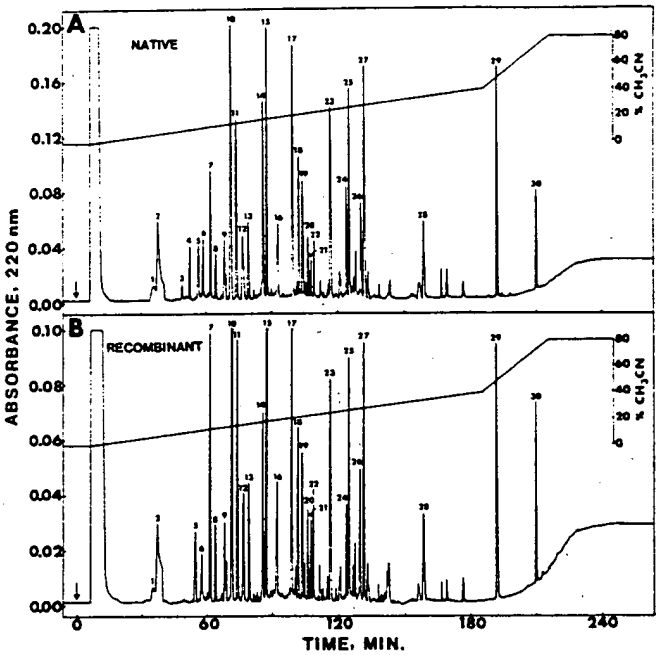

FIG. 7. Reverse-phase HPLC profiles of the tryptic digestion of carboxymethylated bGHs. A, bGH (5882-84); B, rbGH-4. Digests were applied to a Vydac C18 column (5 μm, 4.6 × 250 nm) equilibrated with 0.1 trifluoroacetic acid, then eluted with a gradient of acetonitrile. Column flow rate was 0.5 ml/min.

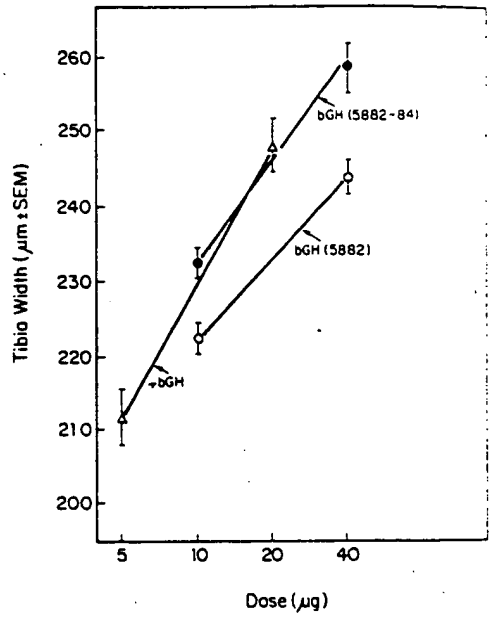

FIG. 8. Dose-response curves for GHs in rat tibia BA. Each point is mean ± SEM of six animals.

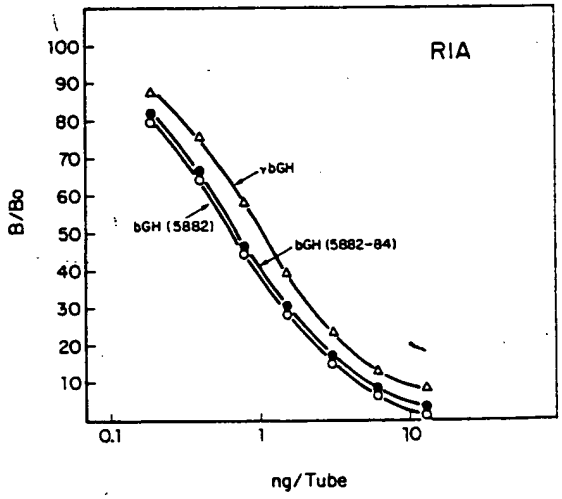

FIG. 9. Displacement curves for bGHs in a homologous RIA: bGH (5882), ○; bGH (5882-84), ●; rbGH-4, △.

and the 45 K variant, which represents aggregates of the hGH. Although the 20 K variant was found to be as active as the 22 K hGH in stimulating body weight gain and somatomedin production in hypophysectomized rats (12, 42, 43), it lacked the insulin-like activity and the lipolytic activity which are characteristic of the 22 K hormone (44). In addition, compared to the 22 K variant, the 45 K aggregates possessed only about 10% of the growth activity and 50% of the lactogenic activity (45). Recently, Hart *et al.* (15) reported that a fraction purified from bovine pituitary extract by passing it through a diethylaminoethyl-Sephacel column possessed growth-promoting activity but was not diabetogenic and possessed little immunoactivity.

Both bGH and hGH have been cloned, expressed, and purified to homogeneity in prokaryotes by various groups (16–18, 25). Protein sequence analysis revealed that the sequence of these bacteria-derived hormones corresponds to the 22 K major form of GH, with an additional methionine at the amino terminus due to the expression vector that was used in these systems. In isoelectrofocusing studies. Stebbing *et al.* (46) found a minor (more acidic) form of bacteria-derived hGH, which they suggested was an artefact generated *in vitro*.

The bGH heterogeneity that we observed may have

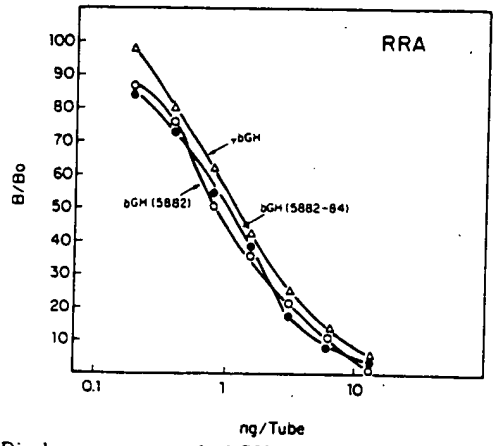

FIG. 10. Displacement curves for bGH in a chicken hepatic RRA: bGH (5882), O; bGH (5882-84), ●; rbGH-4, Δ.

resulted from the fact that the LTR-bGH which we transfected into the mouse fibroblasts was mutated. Doehmer *et al.* (47) reported that the rGH secreted by mouse cells transfected with a Moloney mouse sarcoma virus rGH DNA had a slightly higher mol wt than native pituitary rGH. These workers also reported that their mRNA was approximately 250 nucleotides larger than its *bona fide* rat pituitary counterpart. However, almost all the variants we found with rbGH preparations corresponded with the native pituitary preparations and had ıe leader sequence cleaved from the pro-GH during synthesis. This suggests that the rbGH synthesized in the eukaryotic cells resembled bGH formed by *de novo* synthesis more than did the rbGH derived from prokaryotes.

It is of particular interest that the amino terminus of the rGH was heterogeneous. It was first found in native pituitary bGH (48, 49), suggesting that the leader sequence of the pro-GH was cleaved with fidelity, leaving the end-terminal residue of the bGH either alanine or phenylalanine. The same heterogeneity of the amino terminus was also found when bovine pituitary RNA was translated in the wheat germ cell system in the presence of microsomal membrane (50). Thus, it suggests that the cleavage of the leader sequence of bGH is intrinsically faithful. Our results demonstrate that mouse fibroblasts, which are not secretory in nature, can synthesize, process, and secrete polypeptides when they are transfected with an exogenous DNA whch is coded for a secretory protein.

Using recombinant complementary DNA (cDNA) probes, Seeburg (51) obtained a nucleotide sequence of a gene, known as hGH-V, encoding a protein that differs from hGH in only 13 positions. The protein, which was produced and expressed by a DNA recombinant consist- of simian virus 40 hGH-V in monkey cells, had very w immunoactivity but seemed to have receptor binding activity similar to that of the native hGH-N gene products (23). These data suggest that the heterogeneity of GH could be due to expression of multiple genes. The data presented here suggest that the heterogeneity of bGH is posttranscriptional and posttranslational. The data also suggest that heterogeneity is all derived from the product of a single gene, rather than from multiple gene expression, since most of that observed in the native preparations could be identified in the rbGH preparations.

The bacteria-derived hGH has been reported to possess both diabetogenic and lipolytic activities (52, 53), suggesting that, along with the growth activity of GH, they are intrinsic in nature. Work is in progress to determine the diabetogenic and lipolytic activities of the rbGH purified from mouse fibroblasts.

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Exhibit C

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Formation of Isoaspartate at Two Distinct Sites during *in Vitro* Aging of Human Growth Hormone*

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***In vitro* aging at pH 7.4, 37 °C causes natural sequence recombinant human growth hormone (rhGH), methionyl rhGH, and human pituitary growth hormone to become substrates for bovine brain protein carboxyl methyltransferase, an enzyme that modifies the "side chain" α-carboxyl group present at atypical isoaspartyl linkages. The substrate capacity of rhGH increased at a rate of 1.8 methyl-accepting sites/day/100 molecules of hormone. Reversed-phase high performance liquid chromatography (HPLC) of trypsin digests of aged rhGH revealed two altered peptides not present in digests of control rhGH. These two fragments, which had the amino acid compositions of residues 128–134 (Leu-Glu-Asp-Gly-Ser-Pro-Arg) and 146–158 (Phe-Asp-Thr-Asn-Ser-His-Asn-Asp-Asp-Ala-Leu-Leu-Lys), contained the majority of the induced methylation sites, 22 and 58%, respectively. Isoaspartate can result from deamidation of asparagine or isomerization of aspartate. Isomerization of Asp-130, the only candidate site in 128–134, was corroborated by coelution of the altered fragment with the synthetic isoaspartyl peptide upon reversed-phase HPLC. Evidence is presented that the altered 146–158 fragment is a mixture of two peptides resulting from deamidation of Asn-149 to form 70–80% isoaspartate and 20–30% aspartate at this position. The position of isoaspartate in the altered 146–158 fragment was deduced from mass spectrometry, which indicated a single deamidated asparagine; from methylation stoichiometry, which indicated only one methylation site; and from automated Edman degradation, which showed an absence of asparagine and a low yield of aspartate at position 149. These results show that isoaspartate formation from both aspartate and asparagine is a significant, and possibly the major, source of spontaneous covalent alteration of rhGH and that enzymatic carboxyl methylation provides a powerful tool for assessing this type of modification.**

Although covalent damage to proteins can be caused by numerous reactions involving the side chains of amino acids and the amide linkages of the polypeptide backbone (Harding, 1985), deamidation of asparagine side chains at specific sites is emerging as a major contributor in the pH range to which proteins are most typically exposed. For example, there is evidence that deamidation of asparagine is primarily responsible for the inactivation of lysozyme (Ahern and Klibanov, 1985), triose-phosphate isomerase (Yuan *et al.*, 1981; Casal *et al.*, 1987; Ahern *et al.*, 1987), adrenocorticotropin (Gráf *et al.*, 1973), and calmodulin (Johnson *et al.*, 1985, 1987a, 1989). In small peptides, deamidation of asparagine proceeds most rapidly when the amino acid sequence favors intramolecular catalysis and the formation of a cyclic imide intermediate (Fig. 1; Bornstein and Balian, 1977; Geiger and Clarke, 1987). The major final product of deamidation of this mechanism contains an isoaspartyl linkage wherein the β-carboxyl group of the aspartate is part of the polypeptide backbone, and the α-carboxyl group is present as an atypical one-carbon carboxylic acid side chain. Isoaspartyl linkages can also be generated in small peptides through isomerization of aspartate (Swallow and Abraham, 1958; Geiger and Clarke, 1987). This reaction, which also proceeds through a cyclic imide intermediate, is much slower than the formation of isoaspartate through deamidation of asparagine (Geiger and Clarke, 1987).

Studies on the hydrolysis of aspartyl β-carboxyl esters in small peptides have shown that the amino acid following the aspartyl residue can have a large effect on the rate of cyclic imide formation, the greatest rates being observed when this amino acid is serine, threonine, or glycine (Bodanszky and Kwei, 1978). In untreated proteins and peptides, evidence for isoaspartate has been found at positions originally occupied by Asn-Gly (Gráf *et al.*, 1971; Kanaya and Uchida, 1986; Haley and Corcoran, 1967; DiDonato *et al.*, 1986; Henderson *et al.*, 1976), Asp-Gly (Haley and Corcoran, 1967; Tatemoto and Mutt, 1981), Asn-Ser (DiAugustine *et al.*, 1987), Asn-Thr (Haley and Corcoran, 1967; Ota *et al.*, 1987), Asp-Thr (Ota and Clarke, 1989), Asn-His (McDonald *et al.*, 1983), Asp-Gln (Ota and Clarke, 1989), and Asp-Tyr (Ota *et al.*, 1987). In addition to sequence factors, the bond angles controlling access of the α-nitrogen of the adjacent amino acid to the β-carbonyl of the asparagine or aspartate (see Fig. 1) should play a role in the rate of cyclic imide formation in proteins with considerable structure (Clarke, 1987; Kossiakoff, 1988; Ota and Clarke, 1989).

Interest in isoaspartate has increased in recent years following the discovery that it is selectively methylated by a protein carboxyl methyltransferase (EC 2.1.1.77) (Aswad, 1984; Murray and Clarke, 1984) which is present in a wide variety of species and cell types (Diliberto and Axelrod, 1976; O'Connor and Clarke, 1985). Protein carboxyl methyltransferase methylates synthetic isoaspartyl peptides with little dependence on the surrounding sequence (reviewed by Aswad and Johnson, 1987), and a broad range of protein serves as substrates

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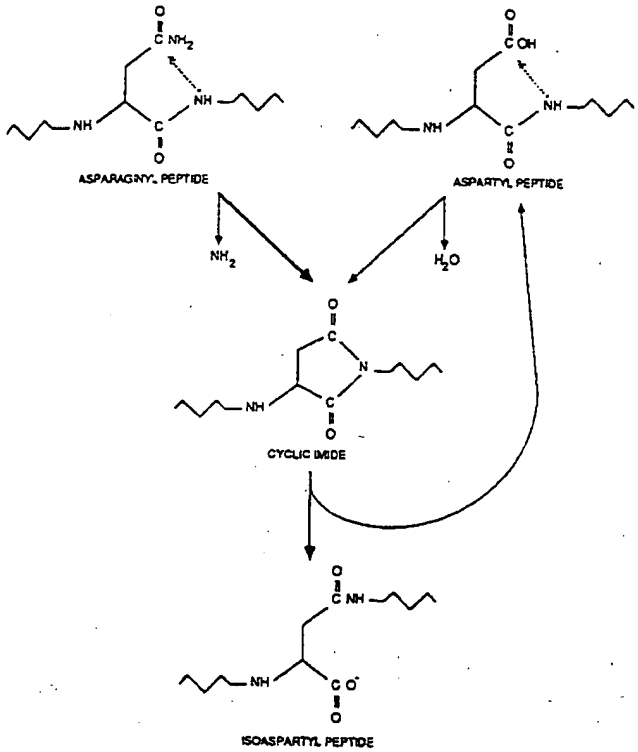

FIG. 1. **Mechanisms for the formation of isoaspartyl linkages upon deamidation of asparagine or isomerization of aspartate.** *Bold lines* indicate the predominant pathways at neutral pH.

both *in vivo* and *in vitro*. The methylation of each protein substrate appears to be limited to a minor subpopulation that presumably contains isoaspartyl linkages. The specificity of protein carboxyl methyltransferase suggests that the enzyme may be involved in the repair or removal of isoaspartate, and the ubiquity of the enzyme may indicate that isoaspartate formation is prevalent *in vivo*. With synthetic isoaspartyl peptides *in vitro*, protein carboxyl methyltransferase can convert a majority of the isoaspartate to aspartate (Johnson *et al.*, 1987b; McFadden and Clarke, 1987; Galletti *et al.*, 1988a). Moreover, this type of conversion has been shown to return activity to isoaspartyl calmodulin (Johnson *et al.*, 1987a).

Methylation by protein carboxyl methyltransferase using the radiolabeled methyl donor *S*-adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet) could provide a simple radiometric assay for assessing the isoaspartyl content of proteins that have become altered during purification, storage, or incubation (DiDonato *et al.*, 1986; Ota *et al.*, 1987; Johnson *et al.*, 1989; Ota and Clarke, 1989). In order to determine the sites that are prone to isoaspartate formation in an intact protein, we have now used protein carboxyl methyltransferase to investigate the occurrence of isoaspartyl linkages in recombinant DNA-derived human growth hormones (rhGH)[1] after aging at pH 7.4 and 37 °C. rhGH is available in two forms, one of which possesses the natural sequence of the hormone, the other of which carries an additional methionyl residue as its amino-terminal amino acid (Met-rhGH) (Kohr *et al.*, 1982). Both forms are effective pharmaceutical agents that are being used clinically for the treatment of hypopituitary dwarfism. hGH is a meaningful model because it is known to become deamidated upon *in vitro* aging at weakly alkaline pH (Lewis *et al.*, 1970; Lewis *et al.*, 1981) and because deamidated forms of the hormone have been detected in human plasma (Stolar *et al.*, 1984; Baumann *et al.*, 1987). A subpopulation of hGH molecules serves as a substrate for protein carboxyl methyltransferase (Diliberto and Axelrod, 1974; Kim and Li, 1979), suggesting that isoaspartyl linkages are indeed formed in the protein. Recently, the deamidation of rhGH was studied by two groups (Hancock *et al.*, 1987; Becker *et al.*, 1988), and it was shown that the asparagine at position 149 was the primary site of deamidation.

Our results show that isoaspartyl bond formation is apparently the major alteration occurring to rhGH during *in vitro* aging under physiological conditions, that isoaspartate can be generated from both aspartate and asparagine in the intact molecule, and that rhGH structure affects the rate of isoaspartate formation, especially at the asparagine site.

EXPERIMENTAL PROCEDURES

Incubations—Recombinant DNA-derived human growth hormones were dialyzed at 4 °C against 50 mM potassium HEPES, pH 7.4, 1 mM EGTA, 5% glycerol, 0.02% sodium azide. Control portions were frozen at −70 °C immediately after dialysis. Aged portions were placed at 37 °C for varying periods of time as indicated. Incubations were stopped by freezing at −70 °C. Incubations of synthetic peptides were performed similarly except that in place of dialysis, the peptides were evaporated from solutions in 0.1% (w/v) trifluoroacetic acid and then resuspended in the aging buffer.

Trypsin Digestions—Aged and control portions of rhGHs (about 2 mg/ml) were dialyzed at 4 °C against 100 mM sodium acetate, 10 mM Tris, 1 mM calcium chloride (pH adjusted to 8.3 with acetic acid). Trypsin that had been treated with L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone to remove chymotrypsin activity (Sigma) was then added to give an initial trypsin to hormone ratio of 1:100 (w/w). After 2 h of incubation at 37 °C, an amount of trypsin equal to the first was added, and digestion was continued for an additional 2 h. In those cases in which digests were to serve as substrates for protein carboxyl methyltransferase, the digestions were stopped by adding phenylmethylsulfonyl fluoride to a final concentration of 4 mM. Otherwise, digestion was stopped by adding 0.10 volume of 88% phosphoric acid.

High Performance Liquid Chromatography—Reversed-phase HPLC of tryptic fragments was performed using a gradient HPLC system from Gilson and 4.6-mm × 10-cm columns of Aquapore RP-300 (Brownlee Labs) fitted with 3-cm guard columns of the same material. Solvent A was 0.1% (w/v) trifluoroacetic acid in water that was purified using a Milli-Q water system (Millipore). Solvent B was 0.08% (w/v) trifluoroacetic acid in acetonitrile. For some experiments, solvent A was 30 mM sodium phosphate, pH 6.5, and solvent B was acetonitrile. All separations employed a linear gradient of 0.5% solvent B/min and a flow rate of 1 ml/min. Detection at 214 nm was accomplished with a Kratos Spectroflow 757 UV absorbance detector.

Methylation Reactions—Intact growth hormones, purified peptides, or phenylmethylsulfonyl fluoride-stopped trypsin digests were methylated in 40-min, 30 °C, pH 6 reactions using the type 1 isozyme of protein carboxyl methyltransferase, which was purified from bovine brain as described previously (Aswad and Deight, 1983). Reactions were carried out in a final volume in which substrates were present at 10 μM final concentration, protein carboxyl methyltransferase at 5 μM (except when protein carboxyl methyltransferase concentration was explicitly varied) and [^{3}H]AdoMet at 200 μM. The specific activity of protein carboxyl methyltransferase was 15–20 nmol/min/mg when 5 mg/ml γ-globulin was used as substrate. The active (*S,S*)-diastereomer of [^{3}H]AdoMet was present at a specific activity of 80–200 dpm/pmol as determined by the method of Hoffman (1986). Methylation reactions containing intact rhGH were terminated by adding 1 ml of 7% (w/v) trichloroacetic acid, and methyl incorporation was determined according to Aswad and Deight (1983). Methyl incorporation into purified peptides was determined using a methanol diffusion assay that is a modification of the method of MacFarlane (1984). Methylation reactions of phenylmethylsulfonyl fluoride-stopped trypsin digests were stopped by freezing at −15 °C. They were thawed

[1] The abbreviations used are: rhGH, recombinant DNA-derived human growth hormone; Met-rhGH, rhGH possessing an additional methionine at the amino terminus; hGH, human growth hormone; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

and immediately injected for reversed-phase HPLC in order to evaluate methyl incorporation into individual fragments.

Amino Acid Compositions—Tryptic fragments (0.2–1 nmol) were collected from reversed-phase HPLC using the trifluoroacetic acid/acetonitrile solvent system. They were then evaporated in acid-washed microcentrifuge tubes by centrifugation under vacuum. HPLC solvent from blank injections was collected in the elution region of each fragment, and these fractions were handled in parallel with the samples. Evaporated samples and blank fractions were resuspended in 0.5 ml of 6 N HCl, 0.1% thioglycolic acid and transferred to Pierce Chemical Co. vacuum hydrolysis tubes. After evacuation, the tubes were heated for 24 h at 110 °C in a block heater. The hydrolysates were transferred to fresh acid-washed microcentrifuge tubes, evaporated, resuspended once in water, and evaporated again. They were then resuspended in 20–60 μl of 2% (w/v) sodium dodecyl sulfate in 0.4 M sodium borate, pH 9.5, evaporated again, and then resuspended in a volume of 12 μM *S*-methylcysteine equal to that of the borate solution. The *S*-methylcysteine served as an internal standard for amino acid composition analysis by the method of Jones *et al.* (1981), which involves derivatization with *o*-phthaldialdehyde followed by reversed-phase HPLC and fluorescence detection of the derivatized amino acids. For the separation of amino acid derivatives, a Beckman Ultrasphere ODS C-18 column was used. Detection was accomplished with a Gilson model 121 fluorimeter using excitation at 305–395 nm and emission at 430–470 nm. Peak areas of amino acid derivatives in the samples were compared with those from a derivatization of a standard mixture of *o*-phthaldialdehyde-reactive amino acids (Pierce Chemical Co.). Amino acids were determined in injections containing 25–100 pmol of derivatized hydrolysate. Hydrolyses were performed in duplicate, and results are reported as the means.

Protein Determinations—Protein concentrations were determined by the method of Lowry *et al.* (1951) following precipitation with 7% (w/v) trichloroacetic acid. Bovine serum albumin was used as a standard.

RESULTS AND DISCUSSION

Generation of Isoaspartyl Methylation Sites in Human Growth Hormone—In order to determine whether significant isoaspartate formation would occur in growth hormone at physiological pH and temperature, Met-rhGH was aged for 14 days at pH 7.4, 37 °C. It was then assayed for its ability to incorporate methyl groups from bovine brain protein carboxyl methyltransferase and [^{3}H]AdoMet. The methyl-accepting capacity was determined by varying the protein carboxyl methyltransferase concentration in reactions containing hormone at 10 μM and [^{3}H]AdoMet at 200 μM. Under these conditions, the methylation should increase with increasing protein carboxyl methyltransferase concentration until it reaches a plateau representing complete modification of the isoaspartyl subpopulation that is accessible to the enzyme (Aswad, 1984). As shown in Fig. 2*A*, a maximal methylation of 0.27 mol of CH_3/mol of Met-rhGH was achieved for the aged protein, whereas the control protein could be methylated to no more than 0.01 mol/mol under the same conditions. Maximal methylation was achieved when protein carboxyl methyltransferase was present at 3 μM or higher. The requirement for a relatively high enzyme concentration is largely due to the fact that this enzyme has an unusually low turnover number. Several previous studies indicate a similar high protein carboxyl methyltransferase requirement for stoichiometric methylation of isoaspartyl peptides or deamidated proteins (Aswad, 1984; Johnson *et al.*, 1985; DiDonato *et al.*, 1986).

Virtually identical increases in methyl-accepting capacity were observed for different lots of Met-rhGH and for natural sequence rhGH from two manufacturers (Table I). Similar experiments have also been carried out on authentic human pituitary growth hormone (Crescormon®, KabiVitrum AB). With this material, the methyl-accepting capacity of the untreated material was quite high (0.17 mol of methyl/mol of hormone) probably because the sample available had been stored under uncontrolled conditions for over 2 years. Nevertheless, a 2-week incubation of Crescormon® under the same conditions used in Table I caused its methyl-accepting capacity to increase to 0.35 mol of methyl/mol of hormone. Although the Crescormon® samples were not studied further, these results show that the aging-induced formation of isoaspartate is not unique to the recombinant DNA-derived forms of hGH.

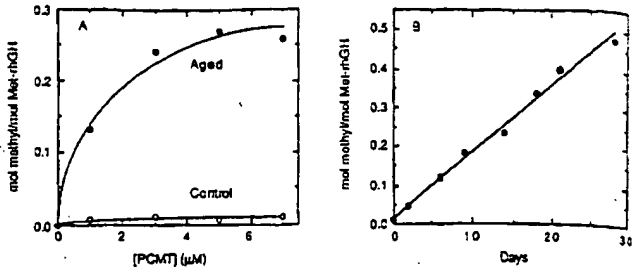

FIG. 2. ***In vitro* aging of Met-rhGH increases its ability to be methylated by protein carboxyl methyltransferase (*PCMT*).** Samples of Met-rhGH (Protropin®) were aged for varying periods of time at 37 °C in 50 mM potassium HEPES, pH 7.4, 1 mM EGTA, 5% glycerol, and 0.02% sodium azide. *A* shows the methyl-accepting capacity of control and 14-day aged Met-rhGH as determined in reactions in which the concentration of hormone was fixed at 10 μM, and the concentration of protein carboxyl methyltransferase was varied. *B* shows the methyl-accepting capacity of Met-rhGH aged for varying times and then methylated at a concentration of 10 μM in the presence of 5 μM protein carboxyl methyltransferase.

TABLE I

Comparisons of aging-induced methyl incorporation into rhGH from various sources

Results are reported as the means of duplicate determinations. The average range of duplicates was 3.1% of the mean for aged samples and 16% of the mean for control samples.

rhGH	Trade name	Manufacturer	Methyl incorporation		
			Control	Aged	Change
			mol/mol		
Met-rhGH[a]	Protropin®	Genentech	0.008	0.269	0.261
Met-rhGH[a]	Protropin®	Genentech	0.005	0.245	0.240
Natural sequence		Genentech	0.004	0.249	0.245
Natural sequence	Humatrope®	Lilly	0.011	0.263	0.252

[a] Two different lots of Protropin® were analyzed.

The time course of the generation of isoaspartyl methylation sites in Met-rhGH is shown in Fig. 2*B*. Methyl incorporation became detectable after 2 days of aging, the earliest time point tested, and it continued to increase over 28 days, approaching 0.5 mol of isoaspartyl methylation sites/mol of polypeptide. The increase in methylation with time was nearly linear in the range studied here, and it proceeded at a rate of 1.8 methyl-accepting sites/day/100 molecules of Met-rhGH. This indicates that hGH is particularly prone to isoaspartate formation. In a recent study, nine proteins were aged under the same conditions used here, and only calmodulin was found to accumulate isoaspartate at a greater rate (Johnson *et al.*, 1989).

Methyl-accepting Tryptic Fragments of Aged Growth Hormone—As a first step in determining the locations of isoaspartate in aged growth hormone, samples of control and 14-day-aged Met-rhGH were digested with trypsin. The digestion mixtures were either injected directly for reversed-phase HPLC using trifluoroacetic acid/acetonitrile solvents, or first methylated with protein carboxyl methyltransferase and [^{3}H]AdoMet and then injected. Peptides were detected by absorbance at 214 nm. Methylated peptides were detected by scintillation counting of collected fractions.

As shown in Fig. 3, *right panels*, there were two major peaks

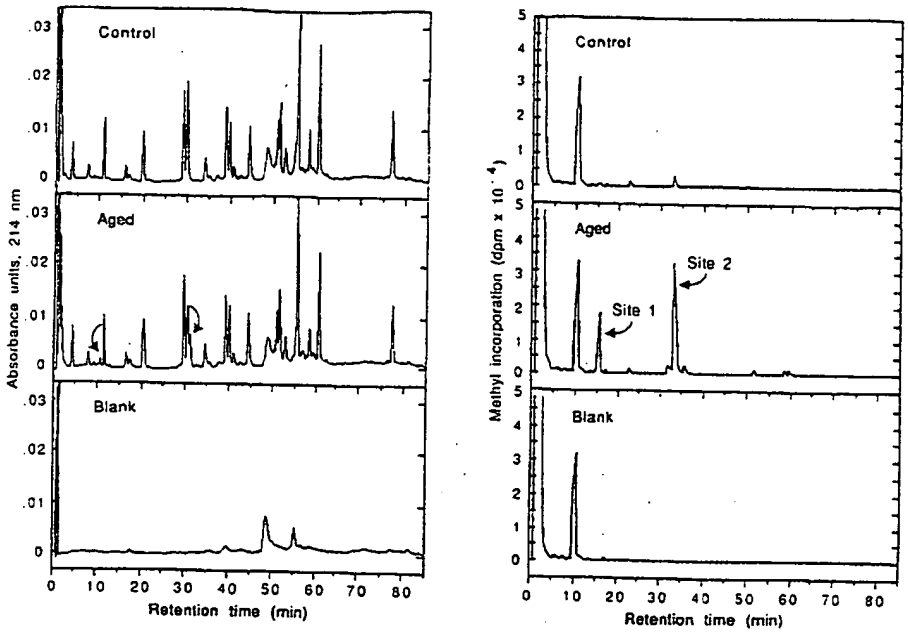

FIG. 3. **Reversed-phase HPLC of tryptic fragments of aged and control Met-rhGH with or without prior methylation by protein carboxyl methyltransferase.** Control Met-rhGH and Met-rhGH that had been aged for 14 days at pH 7.4, 37 °C were digested with trypsin as described under "Experimental Procedures" and were then subjected to reversed-phase HPLC using trifluoroacetic acid/acetonitrile solvents. The *left panels* show the profiles of absorbance at 214 nm for digests of the aged and control material as well as for a blank trypsin digestion mixture lacking hormone substrate. The *arrows* indicate aging-induced changes in the elution of tryptic fragments. The *right panels* show the radioactivity profiles obtained when similar digests were first methylated at a concentration of 10 μM using 5 μM protein carboxyl methyltransferase and 200 μM [^{3}H]AdoMet (200 dpm/pmol). The fraction size was 0.5 ml. The methylation reaction containing a blank trypsin digest as substrate shows that the two large peaks of radioactivity eluting early in the runs are not related to methylation of Met-rhGH fragments. All injections contained the equivalent of 1.2–1.4 nmol of Met-rhGH.

containing methyl groups when the tryptic digest of aged Met-rhGH was used as a substrate for protein carboxyl methyltransferase. The peaks of radioactive methyl groups were much smaller when the tryptic digest of control Met-rhGH was used as the substrate, indicating that the formation of isoaspartate occurred during the 14-day, pH 7.4, 37 °C aging of the Met-rhGH, not during the trypsin digestion procedure. The first major methylated fragment (*site 1*) eluted at 15 min and accounted for 22% of the aging-induced methylation recovered from the column. The second major peak of radioactivity (*site 2*) eluted at 33 min and accounted for 58% of the recovered aging-induced methylation. There were a number of other methylated peptides that were more prevalent after aging of the Met-rhGH. None of these minor methylation sites accounted for more than 5% of the total recovered methyl esters. One minor peak of radioactivity, eluting at 22.5 min, seemed to be attributable to methylation of Met-rhGH, but it did not increase with aging. The sum of these minor peaks made up 20% of the recovered methyl groups.

The total aging-induced methylation of the tryptic fragments recovered from the column represented 0.35 mol of CH_3/mol of injected Met-rhGH. This value, when compared with the 0.26 mol of CH_3/mol of change in methyl incorporation obtained for the intact protein, suggests that all of the methyl-accepting capacity of the intact Met-rhGH can be accounted for by the methylated fragments recovered from the column. The higher methyl-accepting capacity of the tryptic digest may indicate that some of the isoaspartate generated in the intact hormone was not accessible to the enzyme due to conformational constraints. Such an effect has been observed in a more extreme form during studies on the methylation of isoaspartate in deamidated bovine seminal ribonuclease (Galletti *et al.*, 1988b).

Each of the two major methylated fragments eluted shortly after a peptide that changed elution position in an aging-dependent manner (indicated by *arrows* in the *center left panel* of Fig. 3). Because methylation of a carboxyl group increases the hydrophobicity of a peptide and thus causes later elution during reversed-phase HPLC (Murray and Clarke, 1984; Aswad *et al.*, 1987; Johnson *et al.*, 1987b; McFadden and Clarke, 1986; Galletti *et al.*, 1988a), these altered fragments probably contain the isoaspartates formed during aging. The changes in these two tryptic fragments are the only large differences between control and aged Met-rhGH which are apparent from the profile of absorbance at 214 nm. Thus, the formation of isoaspartate may be the major covalent alteration of rhGH occurring under physiological conditions.

In order to test the possibility that the amino-terminal methionine of Met-rhGH affected the rate of formation of isoaspartate at any of the sites, the above experiments were repeated with natural sequence rhGH. The methylated peaks in aged natural sequence rhGH were found to be identical in position and relative amounts to those in aged Met-rhGH (not shown). Absorbance profiles of trypsin digests of control and aged natural sequence rhGH also showed the same aging-dependent shifts in the elution of peptide fragments as did Met-rhGH.

Isolation and Characterization of Methyl-accepting Site 1—The fragment that eluted at 12 min in digests of control Met-rhGH was decreased in area upon aging of the Met-rhGH, and there was a peak at 11.2 min in digests of aged Met-rhGH which was not present in digests of the control material (Fig. 3). Methylated site 1 eluted slightly later than this altered fragment, suggesting that the 11.2-min peak may be the

isoasparty] peptide responsible for site 1. To verify that the 11.2-min peak was the methyl acceptor and not some other peptide preceding methylated site 1, 13 nmol of aged Met-rhGH was digested with trypsin and injected for reversed-phase HPLC using trifluoroacetic acid/acetonitrile solvents. Each peptide fragment eluting before 15 min was collected, reduced to dryness by centrifugation under vacuum, and resuspended in 150 μl of water. Samples of 25 μl each were then assayed for methyl-accepting capacity. As shown in Table II, only the aging-induced peak eluting at 11.2 min exhibited appreciable methyl incorporation.

When the purified 11.2-min peptide was injected for HPLC following methylation by protein carboxyl methyltransferase and [^{3}H]AdoMet, the radiolabeled methyl ester eluted at the same retention time as methylated site 1 (Fig. 4). Therefore, the 11.-2 min peptide did indeed contain methyl-accepting site 1. Fig. 4 also shows that methylation resulted in a complete loss of the original peptide, which indicates that it was quantitatively methylated by protein carboxyl methyltransferase. The methylated product contained 1.06 mol of CH_3/mol of peptide (the amount of peptide was calculated from the absorbance of the methylated peptide at 214 nm relative to the absorbance of the 12-min peptide in an injection of a trypsin digest of 1 nmol of control Met-rhGH). Methylation of the 11.2-min peptide also resulted in the formation of a UV-absorbing unlabeled peak eluting at 13.5 min (Fig. 4*B*). This peak is probably the cyclic imide intermediate that occurs during nonenzymatic demethylation of methylated isoaspartate (Johnson and Aswad, 1985). Imide-containing peptides generally elute between isoaspartyl peptides and their methyl esters under the HPLC conditions used here (Aswad *et al.*, 1987; Johnson *et al.*, 1987b).

The purified methyl-accepting site 1 was subjected to acid hydrolysis, and its amino acid composition was determined using precolumn derivatization with *o*-phthaldialdehyde followed by reversed-phase HPLC (Jones *et al.*, 1981). The 12-min peak, which decreased in area upon aging (see above), was also subjected to amino acid composition analysis. The compositions of the two peptides were identical (Table III) and identified them as a tryptic fragment known to be produced upon digestion of Met-rhGH (Kohr *et al.*, 1982). This fragment contains residues 128–134 of natural sequence hGH, and it has the sequence Leu-Glu-Asp-Gly-Ser-Pro-Arg.

Because Asp-130 is the only site capable of forming isoaspartate in this fragment, it was concluded that the isoaspartate in site 1 was produced by isomerization of Asp-130 in the intact growth hormone. The identity of methyl-accepting site 1 as Leu-Glu-isoAsp-Gly-Ser-Pro-Arg was further strengthened by synthesizing this peptide and showing that it coeluted exactly with the 11.2-min peak present in trypsin digests of aged rhGH (Fig. 5).

Isolation and Characterization of Methyl-accepting Site 2—The fragment of control Met-rhGH eluting at 30 min was decreased in size in the digest of aged Met-rhGH, and the digest of aged Met-rhGH contained a new peak eluting at 31 min (Fig. 3). Methylated site 2 eluted slightly later than this aging-induced peptide (Fig. 3), suggesting that the 31-min peak may contain the isoaspartate responsible for methylated site 2. In order to verify this, the three peaks (29, 30, and 31 min) eluting prior to methylated site 2 were collected from the same 13-nmol injection of aged Met-rhGH trypsin digest which was used for characterization of site 1. Because of the extensive overlap between the 30- and 31-min peaks (see Fig. 3), they were further purified by reversed-phase HPLC using 30 mM sodium phosphate, pH 6.5, acetonitrile solvents. Fig. 6 shows that there were one major peptide and a number of minor peptides present in each of the two peaks collected from the trifluoroacetic acid/acetonitrile solvent system. The peak eluting at 29 min in the trifluoroacetic acid/acetonitrile solvents eluted as a single peak in the pH 6.5 solvent system and was therefore not purified further.

TABLE II

Methyl incorporation into peptides eluting before methylated site 1 in aged Met-rhGH

Retention time	Incorporation
min	*pmol CH_3/25 μl*
8.5	2.9
10.2	0.3
10.8	1.6
11.2	117.8
12.0	5.4

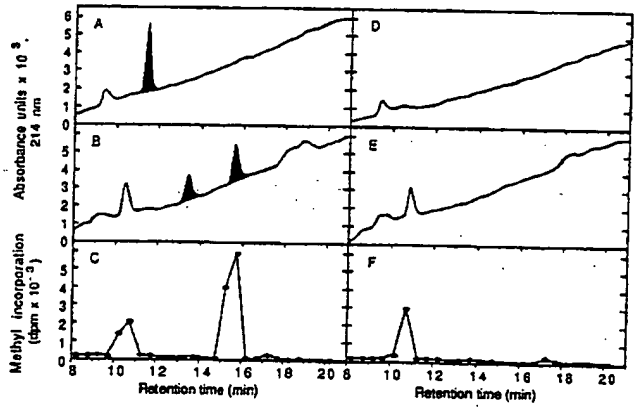

FIG. 4. **Reversed-phase HPLC of purified methyl-accepting site 1.** *Panel A* shows the UV absorption profile obtained with purified site 1 (the 11.2-min peak seen in Fig. 3, *middle left panel*). *Shading* indicates that the 11.2-min peak is not present in a solvent blank run (*panel D*). *Panel B* shows the UV profile of site 1 after methylation with protein carboxyl methyltransferase and [^{3}H] AdoMet. This should be compared with the UV profile of a methylation reaction blank (−peptide) shown in *panel E*. *Panel C* shows the radiolabeling profile corresponding to *panel B*, whereas *panel F* shows the labeling profile corresponding to *panel E*. These results show that site 1 is stoichiometrically methylated since the UV peak in *panel A* is absent in *panel B*. The 15.7-min peak in *panel B* is the methylated form of site 1 since it is radiolabeled (*panel C*). Note that this peak has the same retention time as the labeled site 1 seen in Fig. 3 (*middle right panel*). The 13.5-min peak seen in *panel B* is presumed to be the cyclic imide form of site 1 resulting from spontaneous hydrolysis of the methyl ester (Johnson, and Aswad, 1985).

TABLE III

Amino acid composition of methyl-accepting site 1

Residue	11.2-min peak	12.0-min peak	hGH (128–134)[a]
Ala	0.0	0.0	0
Arg	1.0	0.7	1
Asx	1.0	1.2	1
Gly	0.9	0.6	1
Glx	1.0[b]	1.0[b]	1
His	0.1	0.0	0
Ile	0.0	0.0	0
Leu	1.0	1.2	1
Lys	0.0	0.1	0
Met	0.0	0.0	0
Phe	0.0	0.0	0
Ser	0.9	0.8	1
Thr	0.0	0.0	0
Val	0.0	0.0	0

[a] This peptide also contains 1 proline, which is not detected by *o*-phthaldialdehyde.

[b] Values are normalized to 1.0 mol of Glx/peptide.

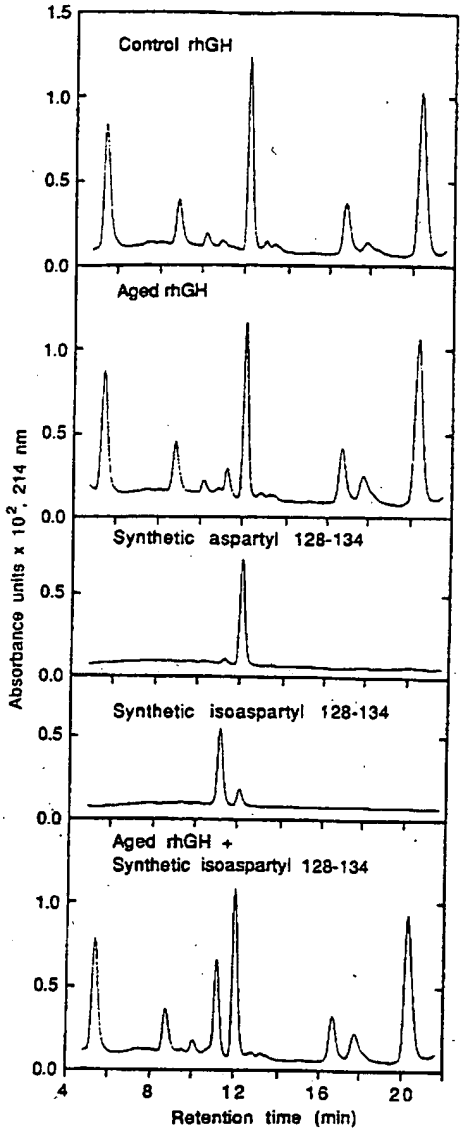

FIG. 5. **Coelution of methyl-accepting site 1 with synthetic Leu-Glu-isoAsp-Gly-Ser-Pro-Arg.** Trypsin digests of 1 nmol of control or aged natural sequence rhGH, or 1 nmol of synthetic aspartyl or isoaspartyl versions of hGH 128–134 were injected for reversed-phase HPLC using trifluoroacetic acid/acetonitrile solvents. In the *bottom panel*, 1 nmol of a trypsin digest of aged rhGH was combined with 500 pmol of synthetic isoaspartyl hGH 128–134 to show exact coelution of the synthetic peptide with the aging-induced peptide eluting at 11.2 min.

Each of the peaks observed during reversed-phase HPLC at pH 6.5 was collected, evaporated by centrifugation under vacuum, and resuspended in 150 μl of water. Samples of 25 μl were then assayed for methyl-accepting capacity. The most abundant peptide in the 31-min trifluoroacetic acid/acetonitrile HPLC peak (peptide 31-b) contained the majority of the isoaspartyl methyl-accepting sites (Table IV), making it a good candidate for methyl-accepting site 2. The other minor methyl-accepting peptides that were separated from the major substrate by reversed-phase HPLC at pH 6.5 (Table IV) may represent isoaspartyl peptides responsible for the two minor peaks of methylation eluting adjacent to site 2 (Fig. 3).

When purified isoaspartyl peptide 31-b was methylated with protein carboxyl methyltransferase and [^{3}H]AdoMet and then injected for reversed-phase HPLC using trifluoroacetic acid/acetonitrile solvents, the methyl ester eluted with exactly

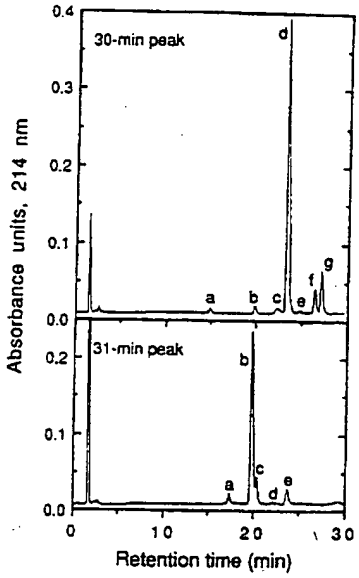

FIG. 6. **Reversed-phase HPLC using a pH 6.5 solvent system for purification of methyl-accepting site 2.** Tryptic fragments of aged Met-rhGH eluting at 30 and 31 min during reversed-phase HPLC in the trifluoroacetic acid/acetonitrile solvent system (see Fig. 3) were collected and subjected to reversed-phase HPLC by the same gradient but using 30 mM sodium phosphate, pH 6.5, as solvent A and acetonitrile as solvent B. The *letters* over the *peaks* signify peptides that were collected and assayed for methyl-accepting capacity.

TABLE IV

Methyl incorporation into peptides eluting before methylated site 2 in aged Met-rhGH

Peptide	Incorporation
	pmol CH_3/25 μl
29	17.5
30-a	2.0
30-b	0.7
30-c	8.0
30-d	2.9
30-e	5.5
30-f	2.4
30-g	3.7
31-a	14.2
31-b	119.5
31-c	20.3
31-d	3.7
31-e	4.0

the same retention time as methylated site 2 (compare Fig. 7 with Fig. 3). The methylated product contained 0.67 mol of CH_3/mol of peptide, suggesting a single methyl-accepting site (the amount of peptide was calculated from the absorbance of the methylated peptide at 214 nm relative to the absorbance of the 30-min peptide in an injection of a trypsin digest of 1 nmol of control Met-rhGH). Methylation of isoaspartyl peptide 31-b also resulted in the formation of an unlabeled peak eluting at 31 min (Fig. 7*B*). This peak is probably the cyclic imide intermediate produced during the demethylation of methylated site 2. A comparison of peaks *A* and *B* in Fig. 7 indicates that methylation of 31-b was not stoichiometric. We believe that the residual material seen at 31 min in band *B* is the result of an asparagine to aspartate conversion at succinimide-prone asparagine sites (Bornstein and Balian, 1977; Blodgett *et al.*, 1985). Indeed, Edman degradation of peptide 31-b (discussed later) indicates that about 20–30% of 31-b contains a normal aspartic acid at position 149.

In order to identify peptides 31-b and 30-d, they were

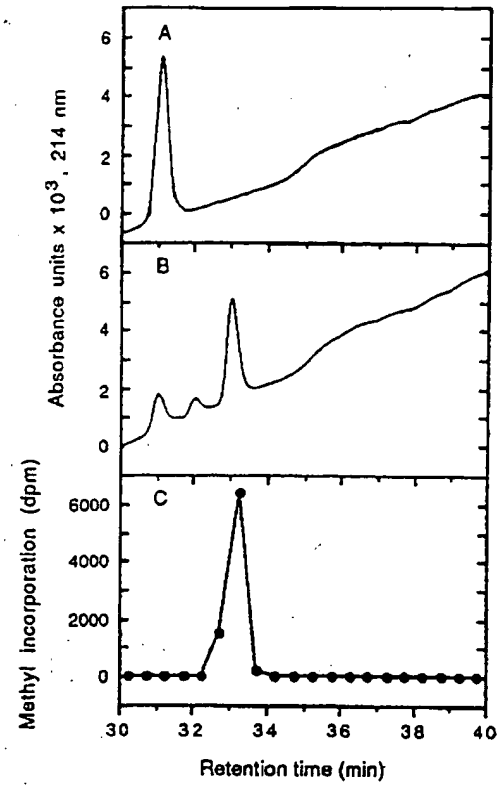

FIG. 7. **Reversed-phase HPLC of purified methyl-accepting site 2.** *Panel A* shows the absorbance profile at 214 nm which was obtained upon trifluoroacetic acid/acetonitrile reversed-phase HPLC of the purified tryptic fragment containing methyl-accepting site 2. The HPLC conditions are the same as used in Fig. 3. *Panel B* shows the absorbance profile obtained when this fragment was first methylated with protein carboxyl methyltransferase and [^{3}H]AdoMet. *Panel C* shows the radioactivity profile obtained when 0.5-ml fractions were collected during the run shown in *panel B* and then subjected to liquid scintillation counting. The UV-absorbing peak eluting between 32.5 and 33.5 min contains radioactivity, indicating that it contains the methyl ester of methyl-accepting site 2. The unlabeled material eluting at 32 min in *panel B* is presumably the cyclic imide that would be generated upon hydrolysis of the methyl ester (Johnson and Aswad, 1985). A blank methylation reaction that lacked peptide substrate gave no peak of radioactivity in the region shown here (not shown).

TABLE V

Amino acid composition of methyl-accepting site 2

Residue	30-min peak	31-min peak	hGH (146–158)
Ala	0.8	0.9	1
Arg	0.1	0.0	0
Asx	4.7	4.7	5
Gly	0.0	0.0	0
Glx	0.0	0.1	0
His	0.7	0.7	1
Ile	0.0	0.0	0
Leu	2.3	2.1	2
Lys	0.3	0.3	1
Met	0.0	0.0	0
Phe	1.2	1.2	1
Ser	0.7	0.7	1
Thr	1.0[a]	1.0[a]	1
Val	0.0	0.0	0

[a] Values are normalized to 1.0 threonine/peptide.

subjected to reversed-phase HPLC using trifluoroacetic acid/acetonitrile solvents, dried, and then subjected to 24-h hydrolysis in 6 N HCl at 110 °C in preparation for amino acid composition analysis. Table V shows that the compositions of isoaspartyl peptide 31-b and peptide 30-d were identical and identifies the fragments as residues 146–158 of natural sequence hGH, which has the sequence Phe-Asp-Thr-Asn-Ser-His-Asn-Asp-Asp-Ala-Leu-Leu-Lys. This tryptic fragment has been observed previously upon digestion of Met-rhGH (Kohr *et al.*, 1982).

There are 2 asparagines and 3 aspartates present in the 146–158 fragment, and therefore the position of the isoaspartyl linkage was not obvious from simply identifying the peptide. The relative elution positions of isoaspartyl peptide 31-b and peptide 30-d in both the pH 6.5 and trifluoroacetic acid/acetonitrile solvent systems were consistent with the identity of isoaspartyl peptide 31-b as a deamidated version of peptide 30-d. Because the side chain carboxylic acid of isoaspartate bears a negative charge at pH 6.5, it should be less hydrophobic than the side chain amide of asparagine. A peptide containing isoaspartate in place of asparagine should therefore elute earlier upon reversed-phase HPLC at this pH. Indeed, in the pH 6.5 solvent system, isoaspartyl peptide 31-b eluted earlier than peptide 30-d (Fig. 6). During trifluoroacetic acid/acetonitrile reversed-phase HPLC performed on the column used in these experiments, synthetic isoaspartyl peptides elute slightly later than the corresponding asparaginyl peptides (Johnson *et al.*, 1987b), and isoaspartyl peptide 31-b eluted later than peptide 30-d (Fig. 3).

In order to establish further the possibility that isoaspartyl peptide 31-b was a deamidated form of peptide 30-d and to determine the number of deamidation sites, peptides 31-b and 30-d were subjected to fast atom bombardment mass spectrometry. Deamidation of a single asparagine residue should result in an increase of 1 atomic mass unit, because NH_2, which has a monoisotopic mass of 16, becomes replaced with OH, which has a mass of 17. The mass expected for the 146–158 peptide is 1489.69 atomic mass units. The positive ion spectra in the molecular ion region for peptide 30-d and isoaspartyl peptide 31-b are shown in Fig. 8. The most abundant ion that was observed for peptide 30-d in the region 1470–1520 *m/z* was 1490.2. The peptide 31-b, the most prominent ion was 1491.2 *m/z*. These results suggest that isoas-

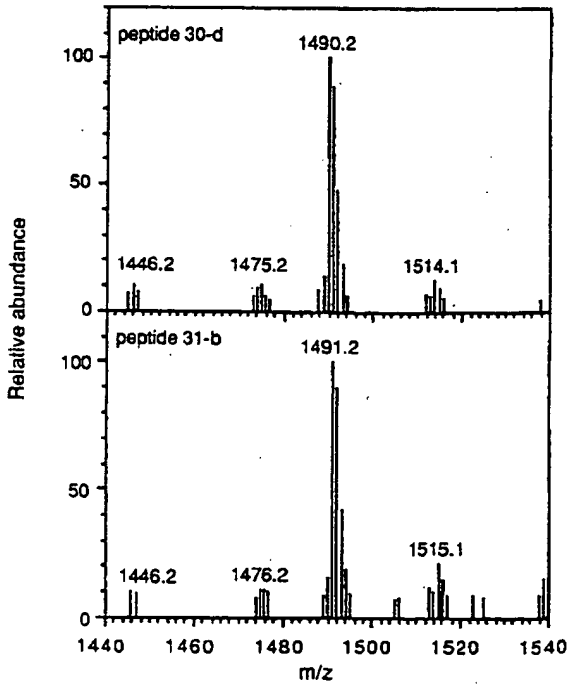

FIG. 8. **Mass spectrum of native and isoaspartyl versions of the 146–158 tryptic fragment from aged Met-rhGH.**

partyl peptide 31-b is a monodeamidated version of the 146–158 peptide.

The location of the deamidated asparagine was detected by automated Edman degradation. Fig. 9 shows the yields of phenylalanine, aspartic acid, threonine, and asparagine through all 13 sequencing cycles with both peptides. In peptide 31-b, no significant asparagine was detected at position 149 (cycle 4), but considerable asparagine was detected at position 152 (cycle 7). The lower relative yield of Asn-152 in peptide 31-b relative to peptide 30-d is expected if one assumes that deamidation of Asn-149 results in a mixture of 75–80% isoaspartate and 20–25% aspartate as suggested earlier in Fig. 7. Edman sequencing fails at isoaspartate (Smyth *et al.*, 1962) but will continue for that portion of the peptide in the aspartyl form. Indeed, the average yields of amino acids in positions 150–158 in peptide 31-b were approximately one-fourth of the corresponding yields obtained with 30-d (data not shown).

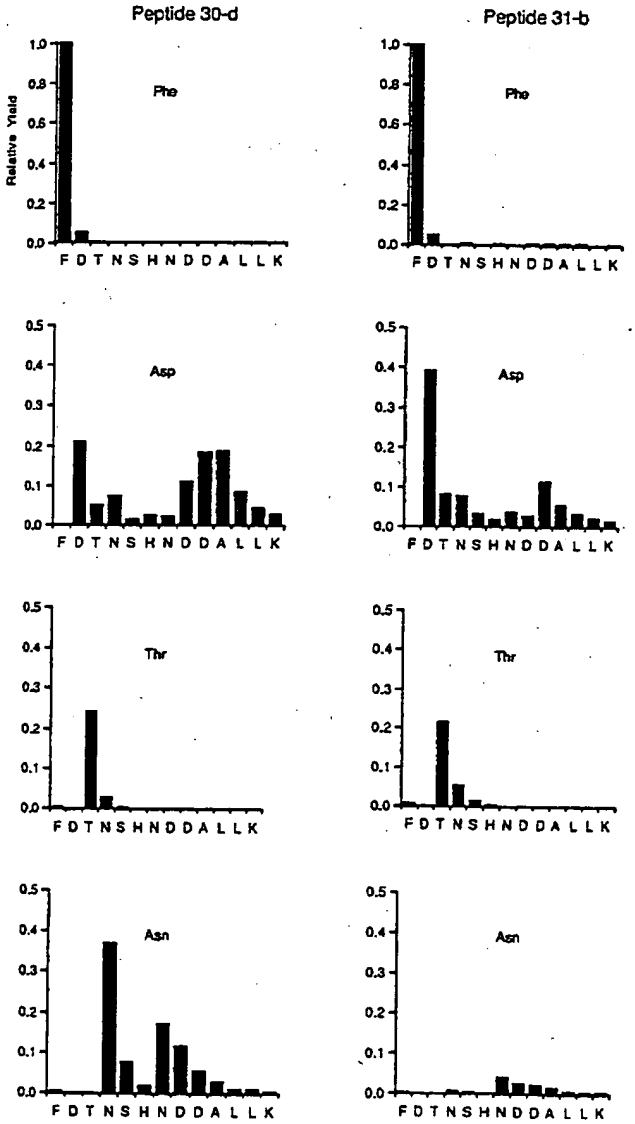

FIG. 9. **Automated Edman degradation of the 146–158 tryptic peptides from native (30-d) and aged (31-b) Met-rhGH.** The abcissa shows the sequence cycle indicated by the single letter code for the residue expected in the native peptide. The ordinate shows the yield normalized to Phe = 1 at the first cycle for both peptides. For simplicity, only plots of phenylalanine, aspartic acid, threonine, and asparagine are shown.

Most importantly, there was no additional drop in relative yield associated with positions 153/154, suggesting the absence of any significant isomerization at these two aspartates. Taken together, the HPLC patterns (Fig. 7), mass spectroscopy data, and the sequencing results are consistent with the hypothesis that site 2 has a single major methylation site associated with the formation of isoaspartate arising via deamidation of Asn-149.

A previous study reported that the major site of asparagine deamidation in hGH was Asn-152 (Lewis *et al.*, 1981). The same study suggested that deamidation of Asn-152 prevented cleavage by subtilisin of the Asn-Ser bond at positions 149–150 (Lewis *et al.*, 1981). Our results suggest another explanation for the altered cleavage of the deamidated hGH: the presence of an isoaspartyl linkage between Asn-149 and Ser-150. The failure of most proteases to cleave isoaspartyl bonds is well documented (Dorer *et al.*, 1968; Pisano *et al.*, 1960; Haley *et al.*, 1966; Haley and Corcoran, 1967; Murray and Clarke, 1984). Our incubations of rhGH at pH 7.4, 37 °C, caused deamidation and isoaspartate formation at Asn-149 but apparently little deamidation of Asn-152. The conditions used in the previous study (Lewis *et al.*, 1981) were somewhat more extreme, employing a pH of 8.3, and they may have resulted in the deamidation of both asparagines with subsequent isoaspartyl bond formation. During the preparation of this manuscript, a report was published by Becker *et al.* (1988) that incubation of rhGH at 37 °C, pH 9, for 72 h resulted in a major site of deamidation at Asn-149 with a minor site at Asn-152. This is consistent with our findings. The additional deamidation observed at the Asn-152 is, again, likely due to the higher pH of incubation used by this latter group.

Influence of Growth Hormone Structure on the Rate of Isoaspartate Formation at Asp-130 and Asn-149—Asn-149 is followed by serine, a sequence that has been shown previously to form isoaspartate in studies on mouse epidermal growth factor (DiAugustine *et al.*, 1987). However, there is another Asn-Ser sequence in rhGH, positions 99–100, which did not appear to form isoaspartate during aging, suggesting that the structure of the intact rhGH molecule significantly influences the rate of isoaspartate formation. Differential degrees of deamidation of asparagine in Asn-Ser sequences have also been observed for trypsin (Kossiakoff, 1988) and were explained by the differing bond angles that control access of the α-nitrogen of the serine residue to the β-carbonyl of the asparagine and therefore dictate the ease of cyclic imide formation.

Although the crystal structure of hGH has not been determined, a moderate resolution crystal structure has been obtained for porcine growth hormone, and the extensive sequence homology between the two hormones allows comparisons to be made (Abdel-Meguid *et al.*, 1987). In the porcine hormone, the stretch of amino acids spanning positions 128–151 is remarkable for its lack of well defined structure (Abdel-Meguid *et al.*, 1987). Hence, there may be considerable freedom of rotation around the peptide backbone in this region, and imide formation may occur easily. This may explain the susceptibility of Asp-130 and Asn-149 to isoaspartate formation. The site in porcine growth hormone corresponding to the Asn-Ser sequence at positions 99–100 in rhGH lies in a short fold between two of the four helices in the molecule (Abdel-Meguid *et al.*, 1987). This region of the molecule might be expected to be more constrained in its movement, thus preventing imide formation.

We were interested in determining the effect of rhGH structure on the amount of isoaspartate formation at Asp-130 and Asn-149. We therefore aged synthetic peptides corre-

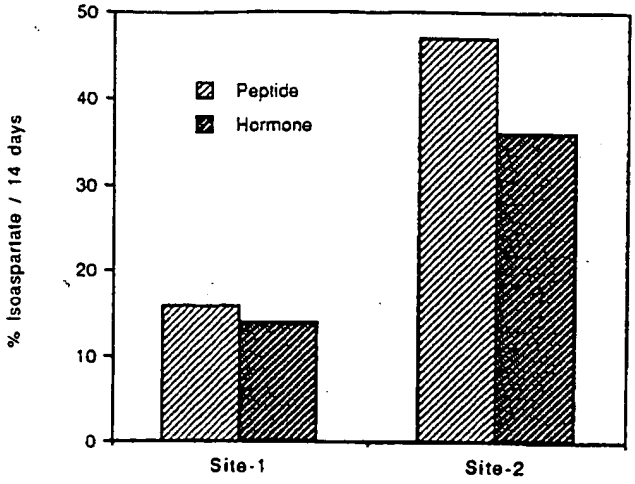

FIG. 10. **Rates of isoaspartate formation at sites 1 and 2 in Met-rhGH compared with the rates of isoaspartate formation in the synthetic peptides corresponding to these sites.** The ordinate indicates the rate as the number of isoaspartates arising per hundred molecules of hormone or peptide after a 14-day incubation at 37 °C, pH 7.4.

sponding to the native 128–134 and 146–158 tryptic fragments under the same conditions that had been used for intact rhGH. Isomerization and deamidation to isoaspartate were evaluated by reversed-phase HPLC in trifluoroacetic acid/acetonitrile solvents. When the synthetic 128–134 peptide was aged for 14 days at pH 7.4 and 37 °C, 16% of the material shifted from 12 to 11.2 min, the elution position of the isoaspartyl peptide. In intact 14-day, pH 7.4, 37 °C aged Met-rhGH, 14% of the 128–134 fragment was present as the isoaspartyl 11.2-min peak (Fig. 5). Therefore, the isomerization of Asp-130 occurred only slightly faster in the small peptide than in the intact rhGH molecule. These extents of degradation correspond to estimated half-lives of 55.6 and 64.3 days for Asp-130 in the synthetic peptide and the intact hormone, respectively. It is interesting to compare these numbers with the data of Geiger and Clarke (1987) who found that a peptide with the sequence Val-Tyr-Pro-Asp-Gly-Ala, *i.e.* with a similar Asp-Gly bond, exhibited a half-life of 53 days when aged *in vitro* at the same pH and temperature. It appears that the Asp-Gly sequence has an inherent half-life of about 53–56 days and that Asp-130 in the intact hormone is in a domain that has a conformational flexibility similar to that of a short synthetic peptide.

When the synthetic 146–158 peptide was aged for the same period of time under the same conditions, 47% of the material shifted to 31 min, the position of the isoaspartyl peptide. In aged Met-rhGH, 36% of the 146–158 peptide was present in the 31-min isoaspartate-containing peptide (Fig. 3). Thus, the rate of deamidation of this peptide was about 50% faster than deamidation in the intact molecule. These results are summarized in Fig. 10. It appears, in this instance at least, that protein structure has an effect on the rate of isoaspartate formation, and the sites that will produce isoaspartate in an intact protein cannot be predicted on the basis of amino acid sequence alone. Bond angles in structured domains of proteins tend to form a large barrier to cyclic imide formation (Clarke, 1987; Kossiakoff, 1988). It is therefore likely that most isoaspartate formation occurs in relatively unstructured domains of intact proteins or in domains susceptible to transient unfolding, so that sequence considerations can come into play.

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Exhibit D

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Crystallization and Preliminary X-ray Characterization of Bovine Growth Hormone

PURIFICATION OF BOVINE PROLACTIN AND GROWTH HORMONE*

(Received for publication, December 27, 1984)

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A new purification scheme for both prolactin and growth hormone from bovine pituitaries has been developed which avoids the use of potentially damaging solution conditions. Both hormones were greater than 95% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and had specific activities similar to or greater than standard samples of the same hormone as judged by several bioassays.

Small single crystals of bovine growth hormone were obtained by vapor diffusion techniques. Examination of these crystals by x-ray diffraction, using the Cornell High Energy Synchrotron Source, showed that they were well ordered, and exhibited diffraction to 2.8-Å resolution on still photographs. Precession and oscillation photographs showed that they belonged to the orthorhombic space group $P2_12_12_1$ (or $P2_12_12$) with unit cell dimensions $a = 219$ Å, $b = 51.9$ Å, $c = 68.9$ Å. The density of the crystals was 1.19 ± 0.02 g/ml from which the presence of eight 45,000-dalton dimers/unit cell was deduced. The protein content of the crystals was shown by isoelectric focusing to be identical to that of purified growth hormone in solution. These crystals appear suitable for use in the x-ray structure determination of bovine growth hormone to at least 3.2-Å resolution.

The two most widely studied hormones from the mammalian anterior pituitary are growth hormone and prolactin. These hormones have molecular weights in the range of 22,000–23,000 and share considerable sequence homology. Growth hormone, also known as somatotropin, is involved in the regulation of growth and metabolism. Prolactin plays a prominent role in the control of lactation.

The determination of the three-dimensional structure of either growth hormone or prolactin would be an important step in understanding the molecular basis of hormone action. Preparations of these hormones from bovine pituitaries have been available for almost four decades (1–3). Yet, despite the efforts of several crystallographic groups, neither hormone (nor the corresponding hormones from any other species) has been obtained in a crystalline form which could be analyzed by x-ray diffraction techniques.

Recent advances have lead to an increased interest in structural studies of these hormones. Growth hormone may have important agricultural applications as an adjuvant to increased productivity in dairy and beef cattle (Refs. 4, 5, and references therein). A similar role for prolactin is possible. Furthermore, the molecular cloning of bovine growth hormone (6) and prolactin (7) provides the means for large-scale commercial production of these hormones. Because of all the above considerations, we decided to re-investigate the crystallization of these hormones.

The crystallization of a protein is often highly dependent on the purity and the method of preparation of the protein sample (8, 9). The available purification procedures for bovine growth hormone and prolactin (Table I) yield protein which was usually described as being highly purified, although in most cases quantitative evidence by which to judge this purity is lacking. Re-examination of samples from some of these preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing convinced us that previous failures in crystallization attempts may have arisen due to inadequate purity of the preparations.

We sought to minimize microheterogeneity in our protein preparation, that is, the presence of several similar but chemically or structurally distinct forms of a protein. Such microheterogeneity may arise naturally if, for example, multiple alleles code for proteins of slightly different sequence. However, it often arises from proteolysis, deamidation, oxidation, or partial denaturation occurring during lengthy purification procedures. Deamidation is especially likely in alkaline solutions. Very high or low pH, detergents, and organic solvents can promote protein denaturation. Most existing procedures (Table I) employ some steps which favor the formation of modified protein forms. Our successful crystallization of the closely related polypeptide hormone, human chorionic somatomammotropin (10), was dependent on the development of a new purification procedure which avoided the use of such potentially damaging conditions. Therefore, we have devised a new, simple method of preparing both hormones which avoids the use of organic solvents and extremes of pH, and which produces pure protein in good yield. This approach has, for the first time, permitted the growth and characterization by x-ray diffraction techniques of crystals of bovine growth hormone.

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§ Recipient of National Institutes of Health Career Development Award AM-00322.

TABLE I

Bovine prolactin and growth hormone preparations

Hormone	Investigators	Reference	Solubilization procedure	Average yield
				mg/g whole pituitary
Prolactin	Jiang and Wilhelmi	12	75% Ethanol, 150 mM NaOH, 3 h, 4 °C[a]	0.8
	Reichert	13	75% Ethanol, pH 10.5, 10 h, 4 °C[a]	1.5
	Kwa *et al.*	14	1% Deoxycholate, pH 8.4, 20 h, 4 °C	2.0
	Bell *et al.*	This work	1 mM EGTA, 10 mM NH_4HCO_3, pH 8.0, 40 min, 37 °C	0.7
Growth hormone	Wilhelmi *et al.*	3	$Ca(OH)_2$, pH 11.5, 24 h, 4 °C	1.6[b]
	Li	11	$Ca(OH)_2$, pH 10.5, 1 h, 4 °C, freeze-thaw	2.0
	Dellacha and Sonenberg	15	$Ca(OH)_2$, pH 11.5, 24 h, 4 °C	0.6[b]
	Kwa *et al.*	14	0.1 M Sodium phosphate, pH 5.5, 16 h, 4 °C, twice	4.0
	Wallis and Dixon	16	Freeze-thaw, 25 mM NaCl, 6.5 mM $Na_2B_4O_7$, pH 8.7, 3 h, 0 °C	1.9[b]
	Reichert	13	250 mM $(NH_4)_2SO_4$, pH 5.5, 16 h, 4 °C[a]	1.0
	Secchi *et al.*	17	250 mM $(NH_4)_2SO_4$, pH 5.5, overnight, 4 °C[a]	1.1
	Bell *et al.*	This work	1 mM EGTA, 10 mM NH_4HCO_3, pH 8.0, 40 min, 37 °C	1.0

[a] Extraction conditions for given hormone, after previous extractions according to Ellis (18).
[b] Yield was originally reported on the basis of weight of anterior pituitary tissue used. A conversion factor of 0.54 g of anterior tissue/g of whole pituitaries was applied.

EXPERIMENTAL PROCEDURES[1]

RESULTS AND DISCUSSION

Through our new purification procedure we have obtained bovine prolactin and growth hormone purer than the most commonly used preparations. Fig. 1 and Table II compare the purity of our preparation with some other standard sources of these hormones. In common with other preparations of bovine growth hormone (19), our preparation contains two forms with different isoelectric points (Fig. 1, *lanes e–h*). This charge heterogeneity has been attributed to the presence or absence of an alanine residue as the NH_2-terminal amino acid (20).

The biological activity of our growth hormone was compared with that of a standard sample of bovine growth hormone from the National Institutes of Arthritis, Diabetes, and Digestive and Kidney Disease in the stimulation of colony formation by Friend virus-infected erythroleukemia cells (21) and the potentiation of erythropoietin-stimulated erythropoiesis of cultured murine bone marrow cells (22, 23). The maximum response observed for all samples in both assays occurred at a growth hormone concentration of 250 ng/ml (Fig. 2). The maximum response from our purest sample was 40–50% higher in these two assays than that of the standard hormone sample. This result might be attributed to the difference in purity between the two samples (Table II). However, impurities in the standard sample might also actively attenuate the response to growth hormone in this assay. At most concentrations, our less pure sample (DEAE-cellulose) also produced a larger response than the standard.

The dose response observed with bovine growth hormone in the erythroleukemia cell line (Fig. 2*a*) was similar to that previously observed with human growth hormone (21). The dose response observed for the murine bone marrow cell culture (Fig. 2*b*) was similar to that previously observed except that previously the maximum potentiation of erythropoiesis occurred at a bovine growth hormone concentration of 100 ng/ml (23).

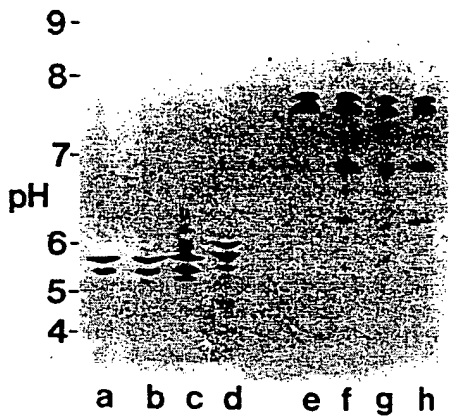

FIG. 1. **Isoelectric focusing gel of samples from various growth hormone and prolactin preparations.** The samples analyzed were: *lane a*, bovine prolactin after preparative electrophoresis; *lane b*, bovine prolactin after DEAE-cellulose column; *lane c*, bovine prolactin obtained from the National Institutes of Arthritis, Diabetes, and Digestive and Kidney Disease, Lot NIH-P-B4 (13); *lane d*, ovine prolactin, gift from Dr. C. H. Li (29); *lane e*, bovine growth hormone after CM-cellulose column; *lane f*, bovine growth hormone after DEAE-cellulose column; *lane g*, bovine growth hormone obtained from Lot NIH-GH-B18 (13); *lane h*, bovine growth hormone, gift from Dr. C. H. Li (11). Each lane contained a total of 10 μg of protein. The approximate pH at the edge of the gel is shown.

We have succeeded in growing single crystals of bovine growth hormone from our purest material. Under most conditions investigated thus far bovine growth hormone has shown a tendency to form polycrystalline aggregates (twins). Nevertheless, under a narrow range of conditions single crystals of growth hormone suitable for x-ray analysis were obtained. The best crystals were grown by the hanging-drop vapor diffusion method (8) from growth hormone solutions

[1] Portions of this paper (including "Experimental Procedures," parts of "Results and Discussion," Figs. 4 and 5, and Table III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-3920, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE II

Purity of bovine hormone samples

Hormone	Source	% Purity*
Growth hormone	This preparation	
	DEAE-cellulose	80
	CM-cellulose	>95
	Lot NIH-GH-B18	65
Prolactin	This preparation	
	DEAE-cellulose	76
	Prep-disc	>95
	Lot NIH-P-B4	72

* Purity was determined as the per cent of total dye binding in that lane that was measured in the main protein band on a sodium dodecyl sulfate-polyacrylamide gel. Gels were heavily loaded with between 4 and 10 μg of protein in order to best detect impurities. Values are the mean of four determinations. Standard deviation was less than ± 4.

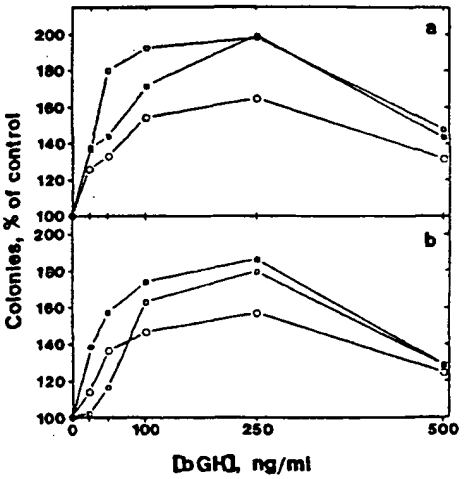

FIG. 2. **The effects of bovine growth hormone on colony formation by Friend virus-infected erythroleukemia cells (*panel a*) and on erythroid colony formation in cultured mouse bone marrow cells (*panel b*).** The results are plotted as a per cent of control values without added growth hormone. Dose-response curves are shown for bovine growth hormone: from DEAE-cellulose (□); from CM-cellulose (■); from Lot NIH-GH-B18 (○, Ref. 13).

with a protein concentration of 2.5–4.8 mg/ml. The hanging-drop contained an initial concentration of 5 or 10 mM potassium TAPS[2] buffer at pH values between 7.6 and 7.8. It was equilibrated at 2–8 °C against the same buffer at 100 mM plus 1–3% ethanol. Crystal growth was aided by seeding with crushed crystals which had been originally grown under similar conditions without seeding. Crystallization occurred over the period of 1 month. Very small needle-like crystals were observed before the formation of rectangular, thin plates.

Fig. 3*a* shows a photomicrograph of bovine growth hormone crystals with the plate habit. The largest crystals had dimensions of approximately 250 × 120 × 10 μm and a volume of 3 × 10^5 μm^3. The crystal that produced the diffraction pattern shown in Fig. 3*b* was a more typical size. Its dimensions were 120 × 120 × 15 μm and its volume was 2.2 × 10^6 μm^3. Analysis of these crystals using high-intensity x-rays at the Cornell High Energy Synchrotron Source, CHESS, required exposures of approximately 30 min for screened precession photographs or 15 min for still and oscillation photographs. Only one photograph could be obtained from each crystal. The crystals were found to be single, and diffracted on still photographs to a resolution of at least 2.8 Å and on 2° oscillation photographs to a resolution of 3.2 Å. Screened precession (Fig. 3*b*) and oscillation photographs showed that the crystals belonged to an orthorhombic space group with cell dimensions a = 219, b = 51.9, c = 68.9 Å. The $hk0$ zone (Fig. 3b) shows systematic absences of the form $h = 2n$ along a^* and $k = 2n$ along b^*; other photographs show $l = 2n$ along c^*, to at least 10-Å resolution. We deduce that the space group is $P2_12_12_1$ (or possibly $P2_12_12$ at higher resolution). These are the most weakly diffracting single crystals of any compound (macromolecule, small organic or inorganic molecule) to have been successfully examined by x-ray diffraction techniques.[3]

These crystals have two unusual features: the long cell dimension along a (Fig. 3*b*) and the large volume of the unit cell (V = 7.83 × 10^5 Å^3). Measurement at 4 °C using a discontinuous Ficoll gradient (24) established the crystal density as 1.19 ± 0.02 g/ml. This crystal density is consistent with the presence in the asymmetric unit of two of the noncovalent hormone dimers found in solution (16, 19). Thus the asymmetric unit may be said to contain a dimer of hormone dimers. Solvent volume of 43% of the total crystal volume and a volume/unit molecular mass ratio of 2.22 Å^3/dalton can be calculated using a partial specific volume of 0.76 ml/g (25). These crystal packing parameters are very close to the average values found for protein crystals (26).

The large volume of the unit cell is a disadvantage of this crystal form because a larger number of x-ray reflections must be measured for structure determination. However, the noncrystallographic symmetry present with two dimers/asymmetric unit may prove to be a major advantage in phase determination (27).

As demonstrated by isoelectric focusing, crystals of bovine growth hormone contain intact growth hormone. Both isoelectrically distinct forms are present in the crystals in approximately the same ratio as they are present in the purified preparation (Fig. 3c).

These crystals are suitable for further structural work leading to the determination of the structure of bovine growth hormone to at least 3.2-Å resolution.

Under conditions which vary only slightly from the range described above, polycrystalline aggregates of needle-like crystals were observed. Some preliminary characterization of these crystals was attempted. Fig. 3*d* shows an electron micrograph of a thin section of these crystals cut perpendicular to their long axis. Assessment of this and other micrographs by optical diffraction suggested an orthorhombic space group. These measurements, together with x-ray diffraction powder patterns of these crystals, were consistent with the space group and unit cell parameters given above for single plate-like crystals (data not shown). However, the possible presence of other crystal forms could not be excluded.

Attempts to crystallize bovine growth hormone obtained from the National Institutes of Arthritis, Diabetes, and Digestive and Kidney Diseases (Lot B18) were unsuccessful. However, the solubility properties of this growth hormone differ from those of our preparation, and different crystallization conditions may be appropriate for this material.

Our description of crystalline bovine growth hormone is the first well substantiated report of a crystalline growth hormone. In 1948 crystal-like objects were described in two preparations of bovine growth hormone (3, 28). The ethanol-precipitated material from these preparations was reported to

[2] The abbreviations used are: TAPS, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}-1-propanesulfonic acid; EGTA, ethylene glycol *bis*(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate.

[3] K. Moffat, D. Bilderback, and W. Schildkamp, unpublished results.

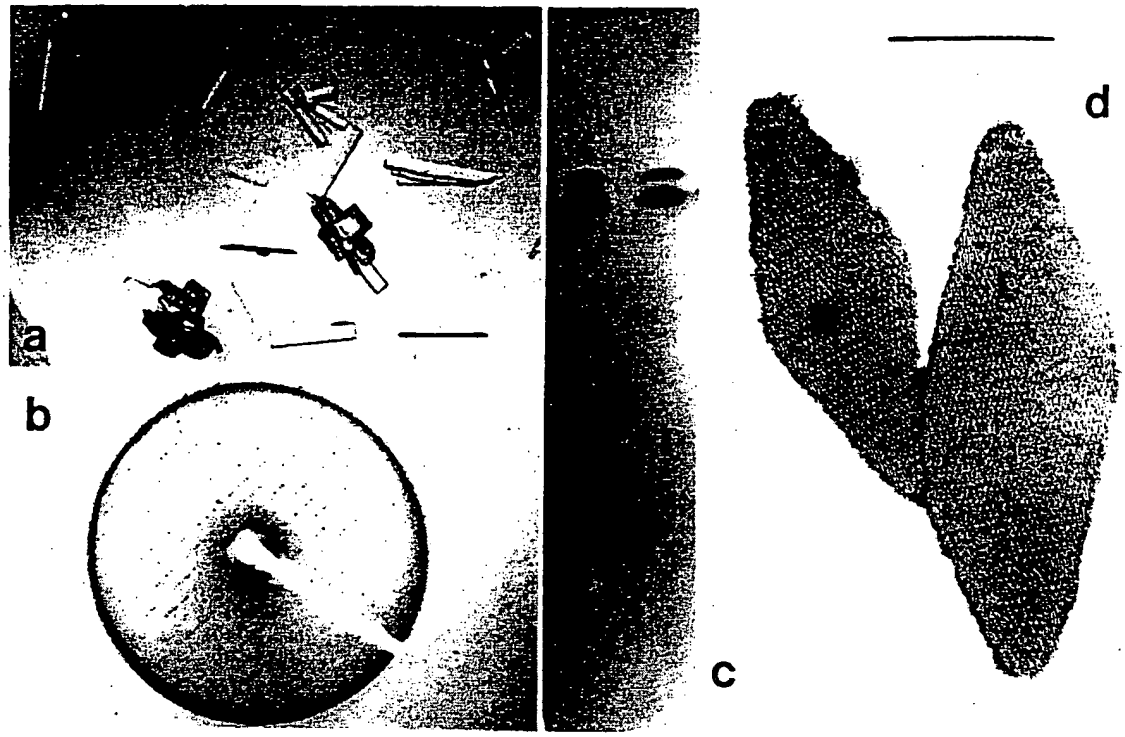

FIG. 3. **Properties of crystals.** *Panel a*, photomicrograph of crystalline bovine growth hormone. The line represents 200 μm. *Panel b*, 10° screened precession photograph of *hk*0 zone of bovine growth hormone. Close spacing of reflections along a^* are equivalent to 1/219 $Å^{-1}$. Resolution at the edge of the photograph is 5 Å. Crystal to film distance was 75 mm. X-ray wavelength was 1.57 Å. *Panel c*, isoelectric focusing gel of dissolved crystals of bovine growth hormone (*left*) and the stock protein solution used for crystallization (*right*). The pH gradient was similar to that shown in Fig. 1. Each lane contained approximately 5 μg of protein. The crystals from a precipitate-free droplet were washed by removing the solution from around the crystals and replacing it with 20 μl of 100 mM KTAPS buffer, pH 7.9, containing 10% polyethylene glycol 6000. This procedure was repeated five times before the crystals were dissolved in 20 μl of 10 mM ammonium acetate, pH 5.5. *Panel d*, electron micrograph of bovine growth hormone crystals. The line represents 200 nm.

have biological activity; however, the identity of the crystal-like objects was not directly investigated. We have not succeeded in obtaining crystals under the same conditions of pH and ethanol concentration as previously reported (3, 28). However, our crystallization conditions are not very dissimilar from, and our results may be related to, these previous reports.

Experiments to crystallize bovine prolactin have not yet been successful but further experiments are planned.

Acknowledgments—We thank Noelle Bersch, Jim Wenban, Theodore Kahn, and Sheri Wischusen for technical help; Margot Szalay and Dr. Christopher Akey for electron microscopy; Dr. Robert E. Hunt for help with x-ray diffraction experiments; Ellen Patterson for manuscript preparation; and Drs. Akey and Hunt for helpful discussion. We thank Dr. C. H. Li for providing samples of bovine growth hormone and ovine prolactin, and Drs. R. L. Noble, C. T. Beer, and P. W. Gout for providing the Nb2 rat lymphoma cell line. We also thank Owasco Meat Co., Moravia, NY, for providing pituitary glands used in preliminary experiments.

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SUPPLEMENTARY MATERIAL
FOR
Crystallization and Preliminary X-ray Characterization of
Bovine Growth Hormone: Purification of Bovine
Prolactin and Growth Hormone
by
Jeffrey A. Bell, Keith Moffat, Barbara K. Vonderhaar and David W. Golde

EXPERIMENTAL PROCEDURE

Material. DEAE-cellulose (DE52) and CM-cellulose (CM52) were purchased from Whatman. Sucrose and ammonium sulfate were ultra-pure grade from Schwarz/Mann. All other chemicals were reagent grade. Bovine pituitaries were shipped on dry ice from Pel-Freez Biologicals and were stored at -60°C until used.

Standard samples of bovine growth hormone and bovine and ovine prolactin were obtained from the hormone distribution program of the NIADDK (National Institutes of Health, Bethesda, MD). Dr. C.H. Li provided additional samples of bovine growth hormone (11) and ovine prolactin (29). Human urinary erythropoietin was obtained from the Blood Diseases Branch, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland.

During the purification of growth hormone and prolactin, all solutions were kept at 0-4° C except as otherwise stated.

Purification Procedure

Granule Isolation. The anterior lobes from approximately 20 partially-thawed bovine pituitaries were dissected away from the diaphragma sellae, posterior lobes and covering connective tissue. They were homogenized in 250 ml of 8% (w/v) sucrose, 2 mM calcium chloride, 1 mM sodium bicarbonate, adjusted to pH 6.0 with HCl (Solution A), using a Waring Blendor at high speed for 1.5 min. The resulting homogenate (Crude, Table 2) was centrifuged at 10,000 x g for 20 min. The pellet was resuspended in 235 ml of Solution A and homogenized further using a Brinkman Polytron with a PT 35 K probe generator for 2 min at a speed setting of 5.5. This homogenate (Polytron, Table III) was layered onto 12 sucrose density gradients in 1 x 3.5 inch polyallomer tubes. Each gradient had the following composition (sucrose concentration expressed in weight per volume): 3 ml 70%, 7 ml of a linear gradient from 70% to 55%, 2 ml 55% and 4 ml 50%. The tubes were centrifuged in a Beckman SW-27 rotor for 20 min at 90,000 x g (max.) in 2 successive centrifuge runs. The material above the 55%-50% interface was discarded and the remaining sucrose layers pooled, diluted with 2 volumes of Solution A and centrifuged at 16,000 x g for 20 min. The pellet was resuspended in 50 ml Solution A (Granules, Table III) and left to stand overnight.

Granule Lysis. The granule suspension was homogenized in a Glenco Scientific Glass-Teflon homogeniser with a 0.01 inch clearance until a smooth homogenate was obtained (about 10 strokes). One liter of 10 mM ammonium bicarbonate, 1 mM EDTA, pH 8.0 was warmed to 37° C and then 0.35 g phenylmethylsulfonyl fluoride and 0.21 g sodium iodoacetate were added. The granule homogenate was slowly poured into this solution with constant slow stirring, and after 40 min incubation the supernatant containing the soluble hormones (Lysate, Table III) was clarified by centrifugation at 16,000 x g for 20 min.

Table III Purification Summary

The course of a typical purification is shown, starting with 35.0 g of whole bovine pituitaries (18.9 g anterior pituitary tissue).

	Total Protein (g)	Growth Hormone			Prolactin		
		Yield (g)	% Yield	% Purity	Yield (g)	% Yield	% Purity
Crude	3.4	0.35	100	10	0.39	100	11
Polytron	2.4	0.34	97	14	0.40	103	17
Granules	1.5	0.30	86	20	0.42	107	28
Lysate	0.43	0.071	20	17	0.080	21	19
DEAE-cellulose		0.060	17	80	0.046	13	76
Prep-disc					0.024	6	>95
CM-cellulose		0.033	9	> 95			

Separation of granule growth hormone and prolactin. 6.64 g of solid ammonium bicarbonate were added to the granule lysate to bring the concentration of ammonium bicarbonate to 90 mM. This solution was loaded on a DE-52 column (4 x 16 cm) equilibrated with 90 mM ammonium bicarbonate, pH 8.0, and eluted with 200 ml of the same buffer. The eluate containing growth hormone (DEAE-Cellulose, Table III) was collected from the first appearance of ultraviolet absorbing material until the eluate had an absorbance of less than 0.2 at 280 nm. Prolactin, which remained adsorbed to the column, was subsequently eluted with a linear concentration gradient of ammonium bicarbonate, pH 8.0, from 90 mM to 180 mM in a total volume of 1.6 l (DEAE-cellulose, Table III). Flow rate for this column averaged 100 ml/h.

Preparative Electrophoresis of Prolactin. Prolactin-containing fractions from the single peak eluted from the DEAE-cellulose column were pooled, lyophilized, and redissolved in 8 ml of 50 mM imidazole-Cl, pH 7.0. Preparative gel electrophoresis was performed on about 25 mg of prolactin as described (10), except that the lower electrode buffer was 100 mM imidazole-Cl, pH 7.0. The absorbance of the eluate was monitored at 280 nm and fractions were pooled from the second and larger of the two peaks eluted (Prep-disc, Table III).

Further Purification of Growth Hormone. Growth hormone from the DEAE-cellulose column was precipitated by adding 313 g of solid ammonium sulfate per liter of eluate. Growth hormone was collected by centrifugation at 12,000 x g for 20 min, redissolved in 30 ml of 5 mM ammonium phosphate, pH 6.5, desalted on a Sephadex G-25 column (2.8 x 38 cm), and loaded on a CM-cellulose column (1.5 x 45 cm) which had been equilibrated with 5 mM ammonium phosphate, pH 6.5. The column was eluted at a flow rate of 15 ml/h with 600 ml of 0.1% (w/v) LKB 7-9 Ampholine titrated to pH 8.40 with KOH (Figure 4). The fractions containing growth hormone were pooled and concentrated by ultrafiltration over an Amicon PM-10 membrane. Growth hormone was separated from Ampholine on a 2.5 x 50 cm Sephadex G-75 column (CM-cellulose, Table III).

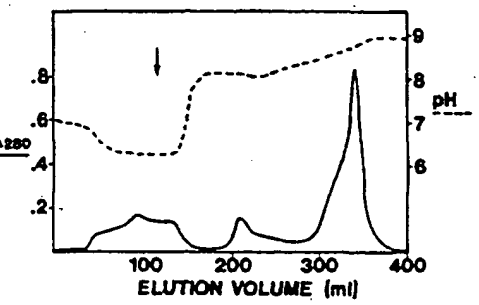

Figure 4. The pH and absorbance at 280 nm of the eluate from the CM-cellulose column, monitored during loading and elution of bovine growth hormone. Elution with the Ampholine solution, as described in Materials and Methods, began at the arrow. Fraction volume was 9 ml. The last peak eluted was growth hormone.

Other Methods

Activity Assays. Differentiation-promoting activity of prolactin was assayed using a mammary explant culture system. Glands from mid-pregnant mice were cultured in serum-free medium for 48 h in the presence of 5 ug/ml porcine insulin, 0.1 ug/ml hydrocortisone, 1 nM 3,5,3'-triiodo-L-thyronine (L-T_3) and various concentrations of prolactins (30). Activity of α-lactalbumin was determined as described (31) using excess exogenous bovine galactosyl transferase (Sigma).

Growth-promoting activity of prolactin on Nb 2 Node rat lymphoma cells was assayed as described (32,33). Cells were cultured for 72 h at 37° C in Fisher's medium containing 10% horse serum and various concentrations of prolactin preparations.

Competition by various prolactin samples with ^{125}I-labelled ovine prolactin for binding to lactogenic receptors on lactating mouse liver membranes was determined as described (34).

The stimulation of Friend virus-infected erythroleukemia cell proliferation by bovine growth hormone was assayed by a microtiter modification of earlier procedures (21,22). Bovine growth hormone samples were diluted in Dulbecco's 1X buffered saline (Irvine Scientific) containing 0.5% bovine serum albumin (Armour Pharmaceutical) and adjusted to pH 7.5 before sterilization by filtering through a 0.2-um Millex filter. The Friend leukemia cell line CM-86 (clone 745) was maintained in continuous suspension culture with Iscove's medium (Irvine Scientific) containing 10% fetal bovine serum. The cells were grown in T-75 flasks for 2 days prior to plating in microtiter dishes for the clonogenic assay. The cells were plated in 0.8% methylcellulose (E4M Premium, Dow Chemicals) with Iscove's medium, 20% fetal bovine serum, 0.1 mM α-thioglycerol and antibiotics using 96-well, flat-bottom microtiter plates (Costar). The final cell concentration was 1 x 10^4 per ml. Hormone samples and diluent controls (without hormone) were added directly to the wells in 10 ul volumes. The microtiter plates were incubated at 37° C in a humidified atmosphere of 9% carbon dioxide and air, and colonies of 8 or more cells were enumerated after 72 h using an inverted microscope.

Potentiation by bovine growth hormone of erythropoietin-stimulated erythropoiesis was assayed in clonogenic murine bone marrow cultures (23,35). Samples were prepared as described above. Bone marrow was obtained from male Swiss-Webster mice and single-cell suspensions were prepared in Iscove's medium containing 20% fetal bovine serum and antibiotics. The bone marrow cells were plated at a final concentration of 2 x 10^5 per ml in lux 1-ml tissue culture dishes with 2-cm grids using 0.8% methylcellulose containing 30% fetal bovine serum, 0.1 mM α-thioglycerol, 0.5 U/ml human urinary erythropoietin. Hormones and diluent controls were added in 0.1 ml volumes. The plates were incubated at 37° C in a humidified atmosphere of 9% carbon dioxide and air for 48 h and erythroid colonies (CFU-E) containing 8 or more hemoglobinized cells were enumerated using an inverted microscope.

Purity Assays. Purity was monitored during the preparation by sodium dodecylsulfate-polyacrylamide gel electrophoresis (36), using a separating gel of 12% (w/v) acrylamide, 0.3% (w/v) bis-acrylamide, in a slab gel apparatus. Samples were added to an equal volume of 0.1 M dithiothreitol, 2.2% (w/v) SDS, 10% (w/v) sucrose, 0.1% (w/v) bromophenol blue, 0.54 M Tris-sulfate, pH 6.1; and incubated in boiling water for 5 min. Gels were stained overnight in 0.25% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol, 10% (v/v) acetic acid, and were destained in the same solution without dye, followed by 5% (v/v) methanol, 10% (v/v) acetic acid. The integrated intensity of staining in protein bands was determined using a Quick Scan gel scanner (Helena Laboratories) with a 525 nm filter. Alternate lanes of each slab gel were run without protein sample to provide an accurate determination of background absorption of the gel.

The quantity of growth hormone and prolactin in crude fractions was determined by comparison of the integrated intensity of the hormone band with the integrated intensity of known amounts of purified hormones on the same gel. At least 3 determinations of each unknown or standard were averaged; replicates varied by less than 15% from the mean. Total protein concentration in crude fractions was determined by the Hartree modification (37) of the Lowry assay (38).

The quantity of hormone in highly purified fractions was determined using $E = 0.71$ $ml.mg^{-1}.cm^{-1}$ at 280 nm for growth hormone (39), and $E = 0.894$ $ml.mg^{-1}.cm^{-1}$ at 277 nm for prolactin (40). A correction for light scattering (41) was made when necessary, based on the absorbance between 320 and 360 nm. The percentage of dye binding in the main protein band on sodium dodecylsulfate-polyacrylamide gel was determined for these fractions as an indication of their purity.

Isoelectric focusing was performed on an LKB Ampholine PAGplate, pH 3.5-9.5, according to the manufacturer's instructions, except that focusing was performed for 0.5 h at 100 V, 0.5 h at 300 V and 2 h at 500 V. Gels were fixed for 5 min in 11.5% (w/v) trichloroacetic acid, 3.5% (w/v) sulfosalicylic acid, rinsed with water, stained for 2 h in several changes of 0.04% (w/v) Serva Blue W in water and destained in water.

Crystallization. Growth hormone to be used for crystallization attempts was equilibrated against 10 mM ammonium acetate, pH 5.5, during the final separation of protein from Ampholine, before concentration by ultrafiltration over an Amicon PM-10 membrane. Prolactin was lyophilized from a 10 mM ammonium bicarbonate solution and dissolved in 100 mM ammonium bicarbonate for use in crystallization experiments. Attempts to crystallize these hormones were made using the hanging drop vapor diffusion method (8). A wide range of crystallization conditions were surveyed between pH 5 and 8.5 using ammonium sulfate, polyethylene glycol 6000, or ethanol as a precipitant.

X-Ray diffraction and electron microscopy. Crystals were mounted in a 0.7 mm glass capillary and exposed to monochromatic x-rays, using a 0.2 mm collimator at the Cornell High Energy Synchrotron Source (42). Diffraction was recorded on Reflex 25 film (CEA). Crystals were cooled to approximately 5° C before and during x-ray exposure.

Crystals were fixed, stained, embedded, and sectioned for electron microscopy as described (43).

RESULTS AND DISCUSSION

Our purification scheme for bovine growth hormone and prolactin is summarized in Table 2. An initial 2 to 3-fold purification of these hormones was achieved by isolation of hormone secretory granules from the anterior pituitary (44), through a procedure modified from the work of Labrie and coworkers (45). Thorough homogenization of the initial crude extract just prior to sucrose density centrifugation is essential to achieve the excellent yield of both hormones in the granule fraction (Table 2). The value of this initial step was first suggested by LaBella et al. (46). Rau et al. (14) previously extracted and separated bovine prolactin and growth hormone from a secretory granule fraction. An important advantage of this approach is that, while the hormones are contained in secretory granules, they are effectively shielded from protease activity in the crude extract.

The presence of secretory granules was a problem as well as an advantage. We have optimized the granule lysis conditions for maximal yield of both growth hormone and prolactin simultaneously ((47), J.A. Bell and T. Kahn, unpublished results). Nevertheless, the lysis step has the lowest yield in our procedure. In a recent study, Moriarty et al. (48) found these secretory granules to be resistant to a wide variety of lysis treatments, except for alkaline pH and 0.5% sodium dodecyl sulfate. Because organic solvents, extremes of pH, or detergents could have undesirable effects on the activity and crystallizability of these hormones, we avoided the use of such conditions for lysis. High pH is known to promote deamidation of asparagine and glutamine residues, a major source of electrophoretic heterogeneity in protein hormone preparations (49). The use of EGTA, elevated temperature, sudden dilution of osmotic support, and slightly alkaline pH was suggested in a study by Labrie and coworkers on the stability of bovine secretory granules containing growth hormone and prolactin (47). The difficulty of solubilizing hormones from these storage granules may explain why more rigorous extraction conditions have often been employed by other investigators (Table 1).

Proteolytic degradation of the prolactin and growth hormone appears to be a problem subsequent to lysis of the granules. Decrease in hormone yields is correlated with the appearance of low molecular weight bands on electrophoresis gels (results not shown). Highest yields were obtained when all steps from granule lysis to elution of prolactin from the DEAE-cellulose column and to loading of growth hormone on the CM-cellulose column were accomplished in one day. Identification of more effective protease inhibitors than those used here (phenylmethylsulfonyl fluoride and iodoacetic acid) might improve hormone yields further .

Chromatography on DEAE-cellulose separated the two hormones and further purified them before final independent purification of each.

Final purification of prolactin was achieved by preparative electrophoresis. This technique has the advantage of high resolution separation on the basis of size and charge (50). The final product was judged to be of very good purity by electrophoresis (Table 3) and by isoelectric focusing (Figure 2). The minor doublet on the acid side of the main prolactin band observed in isoelectric focusing gels (Figure 2, lane a) may be due to deamidation (49). A single protein band was observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a single monomer peak was observed on gel exclusion chromatography (results not shown).

The high resolution technique of chromatofocusing was employed for the final purification of growth hormone (Figure 4). This technique employs a pH gradient during elution which allows the separation of proteins on the basis of their isoelectric points (51,52). The final product was judged to be of excellent purity by electrophoresis (Table 3) and isoelectric focusing (Figure 1). As reported by others (reviewed in Refs. 16 and 19), bovine growth hormone behaved as a dimer on gel exclusion chromatography (results not shown).

The final yield of growth hormone and prolactin from our preparation appears to be somewhat lower than that from some other preparations (Table 1). However, these yields are based on the total amount of protein recovered, not on the amount of prolactin or growth hormone obtained. When considering the yields from these procedures, the purity of the preparation should be considered (Table III and Figure 2). Therefore, our yields could be similar to or better than those obtained by these other procedures.

In three different assays we compared the biological activity of two fractions from our preparation of bovine prolactin to that of two different lots of standard ovine prolactin from NIADDK. The stimulation of α-lactalbumin synthesis in mammary gland explants (30,31, Figure 5a), the stimulation of growth in Nb 2 Node rat lymphoma cells (32,33, Figure 5b) and the competition for binding to lactogenic receptors (34, not shown) were examined. Overall, the four prolactin samples had similar potency. A more detailed account of these results is given elsewhere (53).

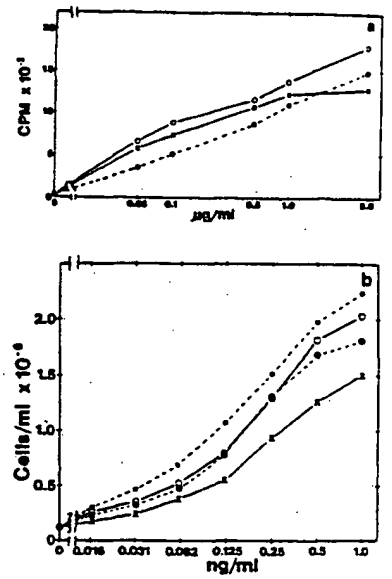

Figure 5. The dose-responses of various prolactin samples for three different biological assays. The samples assayed were: bovine prolactin from the DEAE-column (●), bovine prolactin purified by preparative electrophoresis (○); NIADDK ovine prolactin, lot NIH-P-S14 (X); NIADDK ovine prolactin, lot NIH-P-S15 (■, ref. 13). In each graph the concentration of prolactin is plotted on the abscissa on a logarithmic scale. Panel a. The differentiation-promoting activity of prolactin samples was assayed by determination of α-lactalbumin activity, which is plotted versus the prolactin concentration. Both NIADDK prolactin samples produced the same results within experimental error so that the results were combined and their symbols are coincident. Panel b. The prolactin-dependent stimulation of growth in Nb 2 cells is shown. Control cultures grown in the presence of 10% fetal calf serum produced 1.2×10^5 cells/ml.

Exhibit E

Comparison of the galactopoietic response to pituitary-derived and recombinant-derived variants of bovine growth hormone

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ABSTRACT

Two studies were designed to examine the differences in galactopoietic potency of molecular variants of pituitary- and recombinant-derived bovine GH (bGH). The recombinant bGH molecules included amino-terminal and position-127 amino acid substitutions which are representative of two of the four natural pituitary variants or of partially degraded bGH molecules. Amino-terminal variants of bGH included methionine (Met^1), alanine (Ala^1), serine (Ser^1) or deletion of four amino acids (Δ^{1-4}). The Δ^{1-4} variants were representative of degradation products previously isolated in pituitary bGH preparations. In the first study, 54 lactating Holstein cows received i.m. injections of a buffer solution (control), pituitary-derived bGH, or recombinant-derived [Met^1,Leu^{127}]-bGH, [Met^1,Val^{127}]-bGH, [Ala^1,Leu^{127}]-bGH, or [Ala^1,Val^{127}]-bGH. Cows received 25 mg bGH/day for 21 days. Substitution of the amino-terminal alanyl residue with methionine did not affect milk response. GH variants with Val^{127} elicited a greater milk response (8·5 kg/day) than Leu^{127} bGH variants (6·5 kg/day). The average milk response to the four recombinant bGH variants was 7·5 kg/day greater than controls compared with 4·4 kg/day for pituitary-derived bGH. In contrast, blood bGH concentrations were equivalent for pituitary and recombinant bGH treatments, approximately 20 μg/l more than control levels at 3 h after injection. Blood free fatty acid concentrations were increased, but insulin and glucose levels were unaffected by bGH treatment. In the second study, 54 lactating Holstein cows received i.m. injections of a buffer control solution or recombinant-derived [Met^1,Leu^{127}]-bGH, [Ser^1,Leu^{127}]-bGH, [Ser^1,Val^{127}]-bGH, [Δ^{1-4},Leu^{127}]-bGH or [Δ^{1-4},Val^{127}]-bGH. Cows received 25 mg bGH/day for 28 days. The milk response to full-length bGH variants was 6·6 kg/day greater than the response to the amino-terminal deletion variants ($P<0·05$). Substitution of valine for leucine did not affect milk response to either the deletion (Δ^{1-4}) or full-length (Met^1 or Ser^1) bGH molecules. In conclusion, the lowered galactopoietic potency of pituitary bGH preparations was demonstrated, at least in part, to be due to the presence of amino-terminal amino acid deletions rather than differences in amino acid sequences of recombinant bGH. Ala^1 bGH variants with valine at postiion 127 elicited a greater milk response than Leu^{127} variants.
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INTRODUCTION

Research conducted in the last four decades has demonstrated that exogenous bovine growth hormone (bGH) increases milk production of lactating dairy cows. Although initial studies utilized pituitary-derived bGH (pbGH), subsequent studies have shown that recombinant bGH (rbGH) also has galactopoietic

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activity. Daily injections of zinc methionyl rbGH ([Met^1,Leu^{127}]-bGH) and pbGH over a 6-day period increased average milk and blood GH concentrations similarly (Bauman, DeGeeter, Peel *et al.* 1982). However, daily injection of 27 mg [Met^1,Leu^{127}]-bGH for 188 days elicited a 36·2% increase in milk yield, compared with only 16·5% for the same dosage of pbGH (Bauman, Eppard, DeGeeter & Lanza, 1985). The bGH preparations used in that study had similar potency in both bovine liver receptor binding and hypophysectomized rat growth assays (Wood, Salsgiver, Kasser *et al.* 1989) but clearly differed in galactopoietic potency. The difference between rbGH and pbGH in cattle was also not explained by differences in blood bGH concentrations, and no significant anti-bGH titres were detected (Bauman *et al.* 1985).

One explanation for the observed differences in milk response between rbGH and pbGH preparations could be the amino acid composition of the bGH administered. The rbGH used in the dairy studies of Bauman *et al.* (1982, 1985) had an amino-terminal methionine which is not present in pbGH. Bovine GH is released from the pituitary as four variants composed of alanine or phenylalanine residues at the amino-terminus and leucine or valine substitutions at residue 127 (Li & Ash, 1953; Seavey, Singh, Lewis & Geshwind, 1971; Charrier & Martal, 1988). Differing relative amounts of the four variants are present because of ambiguous processing of the bGH precursor (amino-terminal variants) or allelic polymorphism (residue-127 variants) within a given animal (Pena, Paladini, Dellacha & Santome, 1969; Seavey *et al.* 1971). Hart, Blake, Chadwick *et al.* (1984*a*) resolved four protein peaks from a pbGH preparation and reported differential metabolic activities for them. If a specific bGH variant or combination of variants present in pbGH is required for optimal milk response, rbGH may not match that configuration and the biological response may be altered.

The objective of this study was to determine the basis for the difference in galactopoietic response to rbGH and pbGH. The rbGH variants tested included amino-terminal variants or deletions and position-127 amino acid substitutions which are representative of two of the four bGH variants which are present in the bovine pituitary or of partially degraded GH molecules.

MATERIALS AND METHODS

The rbGH variants were produced individually in *Escherichia coli* K12 W3110G via a pBR322 plasmid (Seeburg, Sias, Adelman *et al.* 1983; Calcott, Kane, Krivi & Bogosian, 1988). The rbGH was purified (Wood *et al.* 1989) to 94–99% homogeneity as judged by reverse-phase high-performance liquid chromatography and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Table 1). The amino acid sequence of all rbGH variants was confirmed by tryptic peptide mapping (Violand, Takano, Curran & Bentle, 1989). All of the peptides from [Met^1,Leu^{127}]-bGH were isolated and their amino acid sequences determined to be correct using an Applied Biosystems model 470A gas phase sequenator (Foster City, CA, U.S.A.). Peptide maps of variants were obtained and peptides which were different from [Met^1,Leu^{127}]-bGH fragments were isolated and sequenced to confirm their identity. The pbGH (Monsanto Lot number M-62-10124) was obtained from A. F. Parlow and characterized by Wood *et al.* (1989). All bGH preparations were non-pyrogenic and sterile as determined by the United States Pharmacopeial rabbit pyrogen and sterility assays (USP, 1990). Relative potencies of the bGH preparations were analysed in hypophysectomized rat growth assays (Marx, Simpson & Evans, 1942). Results ranged from 1·32 to 1·63 units/mg protein (Table 1) with an average index of precision (λ) of 0·41.

TABLE 1. Characterization of bovine growth hormone (bGH) variants utilized for experiments 1 and 2

bGH preparation	Rat growth (units/mg protein)	SDS-PAGE profile (% monomer)
Experiment 1		
pbGH	1·49 (1·38, 1·61)	94·0
[Met^1,Leu^{127}]-bGH	1·57 (1·44, 1·72)	98·5
[Met^1,Val^{127}]-bGH	1·47 (1·37, 1·58)	98·5
[Ala^1,Leu^{127}]-bGH	1·63 (1·49, 1·78)	98·2
[Ala^1,Val^{127}]-bGH	1·57 (1·44, 1·72)	98·7
Experiment 2		
[Met^1,Leu^{127}]-bGH	1·32 (1·26, 1·38)	99·4
[Ser^1,Leu^{127}]-bGH	1·56 (1·43, 1·71)	98·9
[Ser^1,Val^{127}]-bGH	1·34 (1·28, 1·40)	98·8
[Δ^{1-4},Leu^{127}]-bGH	1·45 (1·34, 1·57)	99·5
[Δ^{1-4},Val^{127}]-bGH	1·35 (1·28, 1·42)	99·4

In each experiment, 54 Holstein cows received either 0 or 25 mg bGH/day (0·04 mg/kg body weight) for 21 days (experiment 1) or 28 days (experiment 2). Preparations of bGH other than pituitary-derived bGH (pbGH) were recombinantly derived. Rat growth was measured in a hypophysectomized rat growth assay (Marx *et al.* 1942). Biopotency and (95% fiducial limits) are presented. The average index of precision (λ) was 0·41.

Animals

Lactating multiparous Holstein cows (approximately 60–200 days postpartum) were housed in a free stall barn and milked twice daily at approximately 05.00

and 17.00 h. Milk samples were collected on days −6, −1 and 7 of study and weekly thereafter. Samples were preserved with $K_2(Cr_2O_7)$ and analysed for fat, protein (Multispec Infrared Analyzer Berwind Instrument Group, York, N. Yorks, U.K.) and somatic cell counts (Coulter Milk Cell Counter, Coulter Electronics, Hialeah, FL, U.S.A.). Milk production was standardized to 3·5% fat content according to the equations of Stoddard (1980). All cows were observed several times daily throughout the study for general health status. Animals were fed a pelleted diet (Purina Mills, St Louis, MO, U.S.A.) formulated to meet or exceed requirements recommended by the National Research Council (1978) of the U.S.A. Feed was offered using a DeLaval Rationmaster II computerized feeding system (Alfa-Laval Agri, Inc., Kansas City, MO, U.S.A.). Alfalfa hay and water were available *ad libitum*. The studies were conducted according to the scientific concepts embodied in the Good Laboratory Practice Regulations (1978) of the U.S. Food and Drug Administration. Guidelines of the National Institutes of Health (1985) of the U.S.A. for the care and use of laboratory animals were followed and the study was approved by the Monsanto Animal Welfare Committee.

Experimental design

Experiment 1

Fifty-four multiparous, lactating Holstein cows (approximately 100 days postpartum) were assigned randomly to one of six treatments: (1) control; (2) pbGH or rbGH; (3) [Met^1,Leu^{127}]-bGH; (4) [Met^1,Val^{127}]-bGH; (5) [Ala^1,Leu^{127}]-bGH; (6) [Ala^1,Val^{127}]-bGH. All growth hormones were prepared as sterile lyophilized powders in sodium bicarbonate and solubilized to 5 g/l (27 mmol $NaHCO_3$/l; pH 9·8). Solutions were freshly prepared daily and refrigerated until used. Cows received 25 mg bGH/day for 21 days (0·04 mg/kg body weight). Control cows received daily injections of buffer (27 mmol $NaHCO_3$/l; pH 9·8). During the 7-day pretreatment period all animals received control injections and were assigned to their respective groups on day 1 of treatment. All injections were into the semitendinosus muscle after the morning milking. Approximately 20 ml blood were collected by tail venipuncture at 3 h after injection on days −6, −1, 1, 7, 14, 21 and 28 of study. It has previously been shown that 3 h after injection coincides with increased bGH concentrations after daily injection (Bauman *et al.* 1982).

Experiment 2

Fifty-four multiparous lactating Holstein cows were assigned to study in a randomized block design. Cows were blocked on stage of lactation which varied from 60 to 200 days postpartum. Cows were housed and cared for as described in experiment 1. Animals received one of six treatments: (1) control buffer or rbGH; (2) [Met^1,Leu^{127}]-bGH; (3) [Ser^1,Leu^{127}]-bGH; (4) [Ser^1,Val^{127}]-bGH; (5) [Δ^{1-4},Leu^{127}]-bGH; (6) [Δ^{1-4},Val^{127}]-bGH (Table 1). The amino-terminal deletion variants (Δ^{1-4}) were representative of degradative products found in some pituitary bGH preparations (Wood *et al.* 1989). Approximately 80% of the Δ^{1-4} bGH had a Ser-Leu-Ser amino-terminal sequence and 20% had Met-Ser-Leu-Ser. Variants with serine substitutions at the amino terminus were also included to gain additional information on the effects of amino acid substitutions at that site. All bGH molecules were prepared as sterile lyophilized powders in sodium bicarbonate and solubilized to 10 g/l (25 mmol $NaHCO_3$/l; pH 10·0). During the 7-day pretreatment period all animals received control injections and were assigned to their respective groups on day 1 of treatment. All bGH-treated cows received 25 mg bGH/day for 28 days. All injections were into the semitendinosus muscle after the morning milking.

Blood analyses

In the first experiment, serum was analysed for GH, non-esterified fatty acids (NEFA), glucose and insulin. GH was measured by double-antibody radioimmunoassay using rabbit polyclonal bGH antibody (lot number R609, Monsanto), goat anti-rabbit γ-globulin (Linco Research Inc., St Louis, MO, U.S.A.) and polyethylene glycol precipitation. The bGH antibody cross-reacts less than 0·1% with bovine prolactin and does not differentiate different bGH variants. ^{125}I-Labelled [Met^1,Leu^{127}]-bGH (Monsanto) was used as the tracer. Intra- and interassay coefficients of variation averaged 11·63 and 5·95% respectively. Recovery of added bGH in serum averaged 105% over the range from 0·7 to 67 μg/l. Insulin was measured by double-antibody radioimmunoassay using guinea-pig anti-bovine insulin (Miles Laboratory, Elkhart, IN, U.S.A.), goat anti-guinea-pig γ-globulin (Antibodies Inc., Davis, CA, U.S.A.) and polyethylene glycol precipitation. ^{125}I-Labelled bovine insulin (Sigma Chemical Company, St Louis, MO, U.S.A.) was used as the tracer. The sensitivity limit of the assay was 18 pmol/l. Intra- and interassay coefficients of variation averaged 14·45 and 11·65% respectively. Recovery of added insulin in serum averaged 99% over the range from 36 to 500 pmol/l. Blood glucose concentrations were analysed using an ACA IV clinical analyser (Dupont, Wilmington, DE, U.S.A.). NEFA were measured using an enzymatic assay (Wako Pure Chemical Industries Ltd, Richmond, VA, U.S.A.).

Statistical analyses

Analysis of variance (ANOVA) for a completely randomized design was conducted using the General Linear Model procedure of the Statistical Analysis System (1985). Blood data were analysed by separate ANOVA for each sampling during the treatment period. The model included treatment and a pretreatment covariate (individual animal deviation from the pretreatment mean (Urquhart, 1982)). When the *F*-test of the main effect of treatment was significant ($P<0{\cdot}05$), contrasts and pairwise comparisons (least significant difference, LSD) were deemed significant at $P<0{\cdot}05$ (Snedecor & Cochran, 1980). Data are expressed as least-squares means ± S.E.M. (Searle, Speed & Milliken, 1980) and *n* (number of animals sampled) unless otherwise stated. One animal in the pbGH group was dropped from study because of persistent inappetence and diarrhoea which potentially confounded its response.

For the second experiment, ANOVA was conducted as described previously except that the model also included stage of lactation (from 60 to 200 days postpartum, divided into three blocks).

RESULTS

Experiment 1

Prior to study, 3·5% fat-corrected milk, milk fat, protein and somatic cell count averaged 25·4 kg/day, 3·51%, 3·12% and 200×10^3 cells/l respectively. Milk production was significantly increased with all bGH treatments beginning in the first week of study and overall (Table 2). Average response to the genetically engineered proteins, except [Ala^1,Leu^{127}]-bGH, was greater ($P<0{\cdot}05$, LSD) than to pbGH. Substitution of the amino-terminal alanyl residue with methionine did not affect milk response, i.e. overall Met = 7·7 kg/day versus Ala = 7·2 kg/day; contrast of [Met^1,Leu^{127}]-bGH and [Met^1,Val^{127}]-bGH versus [Ala^1,Leu^{127}]-bGH and [Ala^1,Val^{127}]-bGH. GH variants with valine at position 127 elicited a greater milk response than their leucine counterparts: Val = 8·5 kg/day versus Leu = 6·5 kg/day; $P<0{\cdot}05$; contrast of [Met^1,Val^{127}]-bGH and [Ala^1,Val^{127}]-bGH versus [Met^1,Leu^{127}]-bGH and [Ala^1,Leu^{127}]-bGH. The response difference between amino-terminal methionyl variants with valine and leucine substitutions at position 127 was less (1·2 kg/day; $P=0{\cdot}81$, contrast) than between alanyl variants (2·8 kg/day; $P<0{\cdot}05$, contrast).

Milk composition was unaffected by treatment (Table 2). Somatic cell counts remained low and were consistent with the low incidence of clinical mastitis. No incidence of elevated rectal temperatures or other clinical indications were observed.

Serum GH concentrations were increased by approximately 20–30 μg/l relative to controls (Table 3). Immunoreactive GH concentrations for the pbGH-treated animals were equivalent to levels achieved in cows receiving rbGH treatments. Glucose and insulin concentrations were not significantly increased by pbGH or rbGH treatments. Blood NEFA concentrations were increased two- to threefold by all bGH treatments (Table 3).

Experiment 2

Pretreatment fat-corrected milk, milk fat, protein and somatic cell counts did not differ among treatment groups and averaged 24·3 kg/day, 3·74%, 3·22% and 191×10^3 cells/l respectively. The clinical health of the animals was excellent during the study, although a transient incidence of mastitis was observed among all treatment groups. During week 4 of the study, three cows receiving [Met^1,Leu^{127}]-bGH exhibited extremely high somatic cell counts, resulting in a significant effect of treatment overall (Table 4). However, only one of the three cows had clinical mastitis and no marked decline in milk production was noted.

Daily injection of bGH significantly ($P<0{\cdot}05$, $F(5,45)$) increased 3·5% fat-corrected milk with no change in milk fat or protein content (Table 4). Milk response to the full-length (191 amino acid) bGH variants was 6·6 kg/day greater than the response to the amino-terminal deletion variants ($P<0{\cdot}05$, LSD). Substitution of valine for leucine at position 127 in either the deletion or full-length bGH molecules did not affect milk response ($P>0{\cdot}05$, contrast of [Δ^{1-4},Val^{127}]-bGH and [Ser^1,Val^{127}]-bGH versus [Δ^{1-4},Leu^{127}]-bGH and [Ser^1,Leu^{127}]-bGH-bGH).

DISCUSSION

The rbGH variants examined in the present study included amino-terminal and position-127 amino acid substitutions which are representative of two of the four bGH variants present in the bovine pituitary (Li & Ash, 1953; Seavey *et al.* 1971). The substitution of valine for leucine had the most profound effect on milk response to [Ala^1,Val^{127}]-bGH and [Ala^1,Leu^{127}]-bGH, two naturally occurring variants. The explanation of this effect is uncertain, particularly since valine/leucine substitution did not affect milk response to Met^1 or Ser^1 bGH.

Although the pbGH and rbGH preparations have similar bioactivity in a hypophysectomized rat assay (Table 1 and Wood *et al.* (1989)), a lower milk response to pbGH than to rbGH has been reported (Bauman *et al.* 1985). The presence of amino-terminal deletions in pbGH preparations was implicated as an important cause for lowered milk response. Comparable pbGH lots from the U.S. National Institutes

TABLE 2. Effect of pituitary- and recombinant-derived bovine growth hormone (bGH) on 3·5% fat-corrected milk production (FCM), milk fat, milk protein and somatic cell count (SCC). Values are means ± S.E.M. for the number of cows in parentheses

			Recombinant bGH			
	Control (12)	pbGH (8)	[Met¹,Leu¹²⁷]-bGH (9)	[Met¹,Val¹²⁷]-bGH (7)	[Ala¹,Leu¹²⁷]-bGH (9)	[Ala¹,Val¹²⁷]-bGH (8)
Response						
3·5% FCM (kg/day)						
Days −6 to 0	25·2 ± 5·12	26·6 ± 2·76	26·0 ± 3·49	24·8 ± 4·74	25·0 ± 3·50	24·6 ± 4·51
Days 1 to 21	25·4 ± 0·68[a]	29·8 ± 0·84[b]	32·5 ± 0·79[cd]	33·7 ± 0·90[d]	31·2 ± 0·79[bc]	34·0 ± 0·84[d]
Milk fat (%)						
Days −6 to 0	3·62 ± 0·437	3·28 ± 0·266	3·41 ± 0·315	3·38 ± 0·639	3·64 ± 0·564	3·66 ± 0·481
Days 1 to 21	3·43 ± 0·100	3·34 ± 0·124	3·40 ± 0·115	3·52 ± 0·131	3·27 ± 0·116	3·42 ± 0·123
Milk protein (%)						
Days −6 to 0	3·13 ± 0·260	3·01 ± 0·145	3·12 ± 0·143	3·03 ± 0·349	3·15 ± 0·139	3·28 ± 0·217
Days 1 to 21	3·28 ± 0·027	3·30 ± 0·034	3·26 ± 0·031	3·31 ± 0·036	3·27 ± 0·031	3·28 ± 0·034
Milk SCC (×10³/l)						
Days −6 to 0	145 ± 78·5	268 ± 304·2	93 ± 25·1	155 ± 86·6	248 ± 301·4	317 ± 374·6
Days 1 to 21	352 ± 80·0	189 ± 97·9	114 ± 92·3	182 ± 104·7	348 ± 92·3	201 ± 97·9

pbGH = pituitary-derived bGH. All bGH treatments were administered as daily i.m. injections at 25 mg/cow per day. Least-squares means adjusted for pretreatment responses. Values within the same row with different superscripts are significantly ($P < 0·05$) different (least-significant difference test).

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TABLE 3. Effect of pituitary- and recombinant-derived bovine growth hormone (bGH) on serum GH, insulin, non-esterified fatty acid (NEFA) and glucose concentrations. Values are means ± S.E.M. for the number of cows in parentheses

			Recombinant bGH			
	Control (12)	pbGH (8)	[Met^1,Leu^{127}]-bGH (9)	[Met^1,Val^{127}]-bGH (7)	[Ala^1,Leu^{127}]-bGH (9)	[Ala^1,Val^{127}]-bGH (8)
Blood response						
Growth hormone (μg/l)						
Days of study: −6, −1	0·2 ± 0·30	0·7 ± 1·51	0·3 ± 0·53	0·5 ± 0·99	0·4 ± 0·51	0·3 ± 0·46
1	1·5 ± 3·01^a	20·4 ± 3·48^b	27·5 ± 3·48^{bcd}	24·3 ± 3·48^{bc}	36·3 ± 3·48^d	32·5 ± 3·69^{cd}
7	0·6 ± 1·70^a	23·6 ± 1·96^{bc}	23·6 ± 1·96^{bc}	19·6 ± 2·22^b	20·7 ± 1·96^b	27·5 ± 2·08^c
14	0·4 ± 2·86^a	22·4 ± 3·30^b	25·5 ± 3·30^{bc}	19·6 ± 3·74^b	31·9 ± 3·30^c	27·1 ± 3·50^{bc}
21	0·6 ± 2·47^a	26·6 ± 2·85^c	22·7 ± 2·85^{bc}	14·8 ± 3·24^b	27·2 ± 2·85^c	21·5 ± 3·03^{bc}
28	0·3 ± 0·39^a	1·1 ± 0·45^a	0·3 ± 0·45^a	2·6 ± 0·51^b	1·0 ± 0·45^a	0·4 ± 0·47^a
Insulin (pmol/l)						
Days of study: −6, −1	149 ± 30·5	170 ± 58·1	164 ± 49·1	169 ± 68·7	178 ± 54·1	163 ± 15·2
1	130 ± 13·3	131 ± 15·4	152 ± 15·4	144 ± 17·4	166 ± 15·4	168 ± 16·3
7	132 ± 18·9	144 ± 21·7	177 ± 21·7	149 ± 24·7	146 ± 21·7	163 ± 23·1
14	150 ± 23·4	163 ± 27·0	199 ± 27·0	194 ± 30·6	199 ± 27·0	182 ± 28·6
21	109 ± 22·7	189 ± 26·3	144 ± 26·3	181 ± 29·8	189 ± 26·3	164 ± 27·8
28	107 ± 12·2	135 ± 15·0	130 ± 14·1	137 ± 16·0	142 ± 141·3	135 ± 15·0
NEFA (μeq/l)						
Days of study: −6, −1	175 ± 103·4	246 ± 110·1	191 ± 131·4	239 ± 250·9	193 ± 58·0	179 ± 81·3
1	135 ± 33·7	254 ± 38·9	223 ± 38·9	154 ± 44·1	222 ± 38·9	203 ± 41·3
7	205 ± 71·4^a	517 ± 82·5^b	645 ± 87·5^b	557 ± 93·5^b	630 ± 82·5^b	660 ± 87·5^b
14	99 ± 47·2^a	351 ± 57·8^b	422 ± 54·5^b	292 ± 61·8^b	444 ± 57·8^b	397 ± 57·8^b
21	112 ± 61·9^a	370 ± 75·9^b	371 ± 75·9^b	383 ± 81·1^b	496 ± 71·5^b	411 ± 75·9^b
28	157 ± 22·7	163 ± 27·8	128 ± 26·2	131 ± 29·7	132 ± 26·2	138 ± 27·8
Glucose (mmol/l)						
Days of study: −6, −1	3·3 ± 0·21	3·3 ± 0·24	3·3 ± 0·24	3·5 ± 0·24	3·4 ± 0·21	3·3 ± 0·14
1	3·6 ± 0·10	3·5 ± 0·10	3·5 ± 0·10	3·5 ± 0·12	3·3 ± 0·10	3·4 ± 0·11
7	4·0 ± 0·09	4·0 ± 0·09	4·0 ± 0·09	4·0 ± 0·11	4·0 ± 0·09	4·0 ± 0·10
14	3·7 ± 0·10	3·8 ± 0·09	3·8 ± 0·09	3·9 ± 0·10	3·9 ± 0·09	3·8 ± 0·10
21	3·8 ± 0·09	4·0 ± 0·10	4·0 ± 0·10	3·9 ± 0·10	3·9 ± 0·09	4·0 ± 0·09
28	3·5 ± 0·10	3·6 ± 0·10	3·6 ± 0·10	3·9 ± 0·11	3·7 ± 0·10	3·7 ± 0·10

pbGH = pituitary-derived bGH. All bGH treatments were administered as daily i.m. injections at 25 mg/cow per day. Least-squares means adjusted for pretreatment responses. Values within the same row with different superscripts are significantly ($P < 0·05$) different (least-significant difference test).

TABLE 4. Effect of recombinant-derived bovine growth hormone (bGH) on 3·5% fat-corrected milk production (FCM), milk fat, milk protein and somatic cell count (SCC). Values are means ± S.E.M. for the number of cows in parentheses

		Recombinant bGH				
	Control (9)	[Met^1,Leu^{127}]-bGH (9)	[Ser^1,Val^{127}]-bGH (9)	[Ser^1,Leu^{127}]-bGH (9)	[Δ^{1-4},Val^{127}]-bGH (9)	[Δ^{1-4},Leu^{127}]-bGH (9)
Response						
3·5% FCM (kg/day)						
Days −6 to 0	24·1 ± 6·48	27·8 ± 6·21	25·7 ± 6·71	22·1 ± 7·87	23·4 ± 7·40	22·9 ± 8·18
Days 1 to 28	23·8 ± 0·68^a	33·5 ± 0·71^c	33·7 ± 0·68^c	33·8 ± 0·68^c	27·2 ± 0·67^b	27·0 ± 0·68^b
Milk fat (%)						
Days −6 to 0	3·89 ± 0·700	3·66 ± 0·422	3·77 ± 0·316	3·69 ± 0·573	3·76 ± 0·592	3·65 ± 0·667
Days 1 to 28	3·58 ± 0·080	3·65 ± 0·080	3·66 ± 0·080	3·84 ± 0·080	3·64 ± 0·079	3·50 ± 0·080
Milk protein (%)						
Days −6 to 0	3·34 ± 0·347	3·17 ± 0·347	3·10 ± 0·146	3·28 ± 0·182	3·28 ± 0·310	3·16 ± 0·292
Days 1 to 28	3·26 ± 0·027	3·22 ± 0·027	3·20 ± 0·027	3·19 ± 0·027	3·21 ± 0·027	3·20 ± 0·027
Milk SCC ($\times 10^3$/l)						
Days −6 to 0	184 ± 185·7	137 ± 71·3	225 ± 362·6	205 ± 132·6	230 ± 265·7	166 ± 139·6
Days 1 to 28	150 ± 69·3	484 ± 68·8	185 ± 68·8	147 ± 69·3	182 ± 68·8	146 ± 68·8

All bGH treatments were administered as daily i.m. injections at 25 mg/cow per day. Least-squares means adjusted for stage of lactation (block) and pretreatment responses. Values within the same row with different superscripts are significantly ($P < 0·05$) different (least-significant difference test).

of Health were previously demonstrated to contain approximately 50% amino-terminal deletions by mass (Wood *et al.* 1989). Although significantly less effective than the full-length variants, the Δ^{1-4} variants did increase milk production by over 3 kg/day compared with that of the controls (Table 4). The milk response to pbGH was 2·7 kg/day less than the response to [Met^1,Leu^{127}]-bGH (Table 2). Milk response to Δ^{1-4} deletion variants was 6·3 kg/day less than the response to [Met^1,Leu^{127}]-bGH. Hypothetically, doubling the mass of bGH deletion variants administered from 50% in pbGH (Wood *et al.* 1989) to 100% in Δ^{1-4} bGH preparations resulted in a 43% decrease in the fat-corrected milk response (2·7 versus 6·3 kg).

A loss of galactopoietic potency caused by deletion of the first four amino acids is consistent with a specific role for the amino terminus in bioactivity. Analysis of the three-dimensional structure of molecules such as methionyl porcine GH indicates that approximately six amino acids at the amino-terminus are easily accessible for intermolecular interactions such as receptor binding (Abdel-Meguid, Shieh, Smith *et al.* 1987). For intact bGH variants, single amino acid substitutions at the amino terminus had less effect than deletions, e.g. Met^1 and Ala^1 molecules elicited similar milk responses (Table 2). Although 80% of the pituitary-derived GH molecules with deletions had serine at the amino terminus (Wood *et al.* 1989), Ser^1 and Met^1 substitutions in full-length bGH variants gave equivalent milk responses (Table 4).

Shorter-term studies have reported equivalent responses to pbGH and rbGH. For example, the 6-day study of Bauman *et al.* (1982) showed similar milk and blood responses to s.c. injections of pituitary bGH (25 mg/day; lot number AFP171) and [Met^1,Leu^{127}]-bGH. A 7-day treatment with pbGH and [Met^1,Leu^{127}]-bGH (0·07 mg/kg body weight) also produced equivalent milk responses in Jersey cows (Heap, Fleet, Fullerton *et al.* 1988). In that study, subtle differences between the two bGH preparations were observed for the ratio of milk yield to mammary blood flow and arteriovenous uptake of acetate. The authors postulated that those differences could have been due to the presence of multiple forms of bGH in the pituitary preparations which were not present in the recombinant bGH (Heap *et al.* 1988).

In experiment 1, the concentrations of immunoreactive GH in the blood of pbGH-treated animals were equivalent to those in cows receiving recombinant bGH molecules despite a twofold difference in milk response. Blood sampling was timed to coincide with the approximate peak GH concentration after injection (Bauman *et al.* 1982). Both pbGH and rbGH variants resulted in similar increases in blood GH after injection (Table 3). One week after treatments were discontinued, concentrations were at or near pretreatment baseline values. Similar blood GH responses were expected for these treatments because [Met^1,Leu^{127}]-bGH and [Ala^1,Val^{127}]-bGH have equivalent half-lives, distribution volumes and total clearance rates in dairy cows (Birmingham, White, Lanza *et al.* 1988). In that and the present study, the radioimmunoassay included a polyclonal antibody against bGH which did not differentiate between endogenous and exogenous GH. Similarly, pbGH (lot AFP 7899C) and rbGH were indistinguishable by radioimmunoassay (Langley, Lai, Wypych *et al.* 1987). Hart, Chadwick, Boone *et al.* (1984*b*) also reported that pbGH (lot number NIH-GH-B2) was immunologically identical with recombinant-derived [Met^1,Leu^{127}]-bGH. Bauman *et al.* (1985) noted that neither pbGH nor [Met^1,Leu^{127}]-bGH stimulated a significant anti-bGH titre in cows injected for 188 days.

In the present study both pbGH and rbGH treatments increased blood NEFA by day 7 (Table 3). Average NEFA concentrations followed the same rank order as average blood GH concentrations (Table 3). In-vivo administration of pbGH and [Met^1,Leu^{127}]-bGH to sheep also increased blood glucose and NEFA concentrations (Hart *et al.* 1984*b*). Exogenous bGH treatment has been demonstrated to chronically enhance the lipolytic response of adipose tissue to homeostatic signals such as adrenaline (Sechen, Bauman, Tyrrell & Reynolds, 1989). When dairy cows are in positive energy balance during bGH treatment, lipid deposition is reduced; cows in negative energy balance mobilize lipids to support the milk response to bGH (Bauman, Peel, Steinhour *et al.* 1988; Sechen *et al.* 1989). Although energy balance was non-estimable in this study, if short-term feed intake was not increased (Bauman *et al.* 1985) the incremental secretion of milk fat alone necessitated synthesis or net mobilization of an additional 0·15–0·30 kg lipid/day (Table 2).

Neither pbGH nor rbGH treatments significantly altered blood insulin or glucose concentrations. Although GH has been demonstrated to increase hepatic gluconeogenesis, decrease whole-body glucose oxidation and decrease insulin sensitivity in dairy cows (Bauman, Dunshea, Boisclair *et al.* 1989), metabolic adaptations for glucose homeostasis often result in no change in blood concentrations.

In the light of the present in-vivo data, care must be exercised in inferring bGH responses between assays and across species. Milk production was higher in the cows treated with rbGH than in those treated with pbGH, and preparations from both sources increased blood NEFA levels. In contrast, in-vitro lipolysis was stimulated by pbGH but not by [Met^1,Leu^{127}]-bGH (Hart *et al.* 1984*b*). Equivalent growth-promoting

activities for several pbGH and rbGH preparations have been reported for in-vivo assays with hypopituitary dwarf mice (Hart *et al.* 1984*b*), mature female rats (Wood *et al.* 1989) and hypophysectomized rats (Langley *et al.* 1987) (Table 1). In-vitro assays of liver receptor binding and cellular differentiation gave equivalent results for pituitary and recombinant bGH (Wood *et al.* 1989). Differences in methods of pbGH extraction from the anterior pituitary probably account for some of the variation among studies. Variation in response could also be due to lack of species homology among the assays. For example, recombinant and pituitary-derived porcine GH stimulated equivalent growth responses when administered to pigs over the dose range 1·5–9·0 mg/day (McLaren, Bechtel, Grebner *et al.* 1990). Variation in bioactivity may also be due to receptor-binding activities of pbGH and bGH variants for different tissues responsible for various metabolic processes. Thus, amino-terminal deletion variants could be less galactopoietic but still stimulate anabolic activity similar to intact bGH molecules. The biological response cascade to exogenous bGH for milk synthesis may proceed through GH receptors with different binding characteristics from those of the receptors through which growth functions are stimulated.

In summary, the lowered galactopoietic potency of pituitary bGH preparations was demonstrated to be due, at least in part, to the presence of amino-terminal deletions (amino acids 1–4) rather than differences in amino acid sequences present in rbGH. Ala^{1} bGH variants with valine at position 127 elicited a greater milk response than Leu^{127} variants. Immunoreactive blood GH concentrations and the resultant NEFA changes in the blood of pbGH-treated animals were equivalent to cows receiving rbGH molecules even though the milk response to pbGH was less than to rbGH.

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Exhibit F

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A comparison of the growth-promoting, lipolytic, diabetogenic and immunological properties of pituitary and recombinant-DNA-derived bovine growth hormone (somatotropin)

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The physiological mechanisms by which growth hormone (somatotropin) exerts its several metabolic activities remain poorly understood. In particular, there is disagreement as to whether the diabetogenic and lipolytic activities of the hormone are intrinsic properties of the molecule or are the result of contamination with other pituitary components. The availability of recombinant-DNA-derived bovine growth hormone (rebGH) presented an opportunity to compare the biological activities of rebGH and pituitary bGH in the absence of pituitary contaminants. Pituitary bGH and rebGH were immunologically identical in the radioimmunoassay for bGH, and good agreement was obtained for the potency of the latter measured by radioimmunoassay (1.6 units/mg) and the dwarf-mouse bioassay (1.4 units/mg). The lipolytic activity *in vitro* was examined by measuring the release of glycerol from rat epididymal fat maintained in the presence of dexamethasone (0.2 μg/ml) and the material to be tested (0.1 and 0.2 mg/ml). Although two preparations of pituitary bGH stimulated a significant ($P < 0.01$) increase in glycerol production, neither rebGH nor recombinant-DNA-derived chicken GH was lipolytic. However, when rebGH was intravenously injected into three sheep (0.15 mg/kg), the increase in plasma non-esterified fatty acids was similar to that measured with the same dose of pituitary bGH. Insulin-tolerance tests were conducted in sheep before and after treatment with rebGH and pituitary bGH (four subcutaneous injections of 0.15 mg/kg). Although the effect of rebGH was less than that of the pituitary hormone, both significantly impaired the ability of insulin to lower the concentration of plasma glucose. These data suggest that the lipolytic and diabetogenic activities of bGH are intrinsic properties of the molecule. However, the lipolytic activity may only become apparent after either modification of the molecule *in vivo* or activation of a lipolytic intermediate.

The expression of bGH with additional *N*-terminal methionine by means of recombinant-DNA techniques has provided a potentially limitless source of the hormone to stimulate milk production in domestic ruminants (Hart, 1983) and has renewed interest in the physiological mechanism(s) by which the hormone achieves this response. Close arterial infusion of bGH into the mammary gland of goats and sheep failed to stimulate galactopoiesis (McDowell & Hart, 1983), and attention has turned to the possibility that the hormone partitions nutrients (glucose and triacylglycerols) away from tissue deposition and makes them available for milk synthesis by exerting its diabetogenic and lipolytic properties (Hart *et al.*, 1978; Hart, 1983; McDowell *et al.*, 1983). However, there has been considerable debate over the question of whether GH is intrinsically lipolytic and/or diabetogenic.

The lipolytic activity of pituitary GH has been recognized since Lee & Schaeffer (1934) dis-

Abbreviations used: GH, growth hormone (somatotropin); bGH, bovine GH; rebGH, recombinant-DNA-derived bGH; hGH, human GH; NEFA, non-esterified fatty acids; SDS, sodium dodecyl sulphate.

covered that growth-promoting extracts of the anterior pituitary depleted fat stored in rats. As purer preparations of the hormone became available, this property of GH underwent considerable investigation to determine the role of GH in fat metabolism [see Goodman & Schwartz (1974) and Rao & Ramachandran (1977) for reviews]. More recently, evidence has accumulated to suggest that the pure hormone may not be lipolytic and that this activity is stimulated by either pituitary contaminants or cleaved forms of GH (Salaman, 1972; Assa & Laron, 1977; Schleyer & Voigt, 1977; Frigeri, 1980; Frigeri *et al.*, 1982, 1983).

Raben & Westermeyer (1952) were among the first to suggest that pig GH could be prepared without diabetogenic activity, and more recently homogeneous forms of hGH and bGH have been prepared which had markedly decreased diabetogenic activity (Lewis *et al.*, 1977; Hart *et al.*, 1984). Lewis *et al.* (1977) partially purified a diabetogenic component from crude hGH ($M_r < 5000$), and furthermore were able to convert non-diabetogenic hGH into a diabetogenic form by proteolytic cleavage, thereby suggesting that the active material is a modified form of GH. This contention is supported by the fact that short sequences of hGH have been found to be diabetogenic in rats and rabbits (Lostroh & Krahl, 1976; Bornstein, 1978).

The availability of rebGH presented a unique opportunity to examine the biological properties of the hormone in the absence of contamination with other pituitary factors and to gain additional information on the heterogeneous nature of the molecule.

Materials and methods

Preparation of recombinant-DNA-derived bovine growth hormone

Bovine GH was expressed in *Escherichia coli* by using a recombinant plasmid (414 Syn, bGH 22) which contained a synthetic copy of the bGH gene under the transcriptional control of an *E. coli* tryptophan promoter (L. Souza, unpublished work).

A 40g batch of the *E. coli* cells containing the rebGH was suspended in 200ml of buffer (20mM-Tris/HCl/50mM-NaCl, pH7.5) and was disrupted by three passages through a Gaulin homogenizer. The broken cells were centrifuged (7000*g* for 15min at 4°C), and the pellet was resuspended in 50ml of distilled water, to which 50ml of acetic acid was added over a period of 10min. The mixture was stirred for 3h and then centrifuged (60000*g* for 30min at 4°C). The rebGH in the supernatant was purified by gel chromatography on a column (5cm × 95cm) of Sephadex G-75 which was equilibrated with acetic acid (10%, v/v). The fractions containing rebGH were pooled, dialysed against distilled water and redialysed against glycine/HCl (0.01M; pH3.6)/lactose (0.2%)/mannitol (0.2%).

The rebGH was similar to preparations of recombinant-DNA-derived hGH in that it possessed an additional methionine residue at the *N*-terminus (Goeddel *et al.*, 1979). Furthermore, it possessed leucine at position 127. The integrity of rebGH was assessed by high-pressure liquid chromatography and SDS/polyacrylamide-gel electrophoresis in the presence and absence of 2-mercaptoethanol (Hart *et al.*, 1984).

Radioimmunoassay

The rebGH was assessed in the radioimmunoassays for ruminant GH (Hart *et al.*, 1975) and prolactin (Hart, 1972) with modifications described by Tindal *et al.* (1982).

Growth-promoting activity

The growth-promoting activity of rebGH was measured with hypopituitary dwarf mice and the method described by Wallis & Dew (1973).

Lipolytic activity (in vitro)

This was examined by measuring glycerol production from rat epididymal fat which had been incubated for 4h in the presence of the test material and dexamethasone (Hart *et al.*, 1984). The activity of rebGH was examined at two concentrations (0.1 and 0.2mg/ml) and compared with two preparations of pituitary bGH prepared by the method of Ellis (1961). A preparation of recombinant-DNA-derived chicken GH (Boone *et al.*, 1983) was included in one of the experiments.

Intravenous injection into sheep

Four groups of three castrated male sheep (23–41kg) were individually housed in metabolism crates and fed on a diet of concentrates and hay *ad libitum*. The sheep were starved for 22h before the start of the experiment, during which they were allowed access to water. On the day of the experiment, three blood samples (6ml) were taken from a jugular catheter, inserted at least 24h previously, and the stability of the pre-injection glucose values was immediately established with a Hypocount blood glucose monitor (Hypoguard Ltd., Woodbridge, Suffolk, U.K.). Each animal was then injected, via the catheter, with 3ml of the test material (0.15mg/kg) followed by 2ml of sterile saline (0.9% NaCl). The treatments were as follows: group 1, glycine/HCl (0.01M; pH3.6)+lactose (0.2%)+mannitol (0.2%); group 2, pituitary bGH dissolved in sterile saline (0.9%, pH11.0), group 3, rebGH dissolved in glycine/HCl (0.01M; pH3.6) + lactose (0.2%) + mannitol

(0.2%); group 4, pituitary bGH dissolved as for group 3. The pituitary bGH had been prepared by the method of Ellis (1961) and its growth-promoting activity (1.6 units/mg) determined by the dwarf-mouse assay. Blood samples (5ml) were collected every 30min for 2h and then at 1h intervals for up to 9h after the intravenous injection.

The plasma was stored at −20°C before analysis for GH (Hart *et al.*, 1975; Tindal *et al.*, 1982), glucose, urea and NEFA (Hart *et al.*, 1978). Only selected samples were analysed for NEFA.

Diabetogenic activity

In this study the diabetogenic activity of rebGH was defined as its ability to impair insulin sensitivity in sheep as measured by the insulin-tolerance test.

Intravenous insulin-tolerance tests were carried out in three groups of four castrated male sheep which had been pre-treated (0.15mg/kg injected subcutaneously twice daily for 2 days) with the test material: group 1, pituitary bGH (1.6 units/mg) dissolved in sterile saline (0.9%, pH11.0); group 2, pituitary bGH dissolved in glycine/HCl (0.01M; pH3.6)+lactose (0.2%)+mannitol (0.2%); group 3, rebGH dissolved as for group 2. The sheep, which had previously been fed *ad libitum*, were starved for 17h before each insulin-tolerance test. On the morning of the test, four blood samples (3ml) were taken at approx. 10min intervals, from a jugular catheter which was inserted at least 24h previously, and the stability of the pre-insulin glucose values was established with the blood glucose monitor. Bovine insulin (0.08 unit/kg, in 2ml of sterile saline, 0.9%) was then injected via the catheter, followed by 2ml of saline. Blood samples were taken at 8min intervals for 32min after the insulin injection, and the plasma was analysed for glucose by using a Chemlab continuous-flow analyser. Each test of experimental material was preceded, 2 days earlier, by an identical control insulin-tolerance test in which the goats had been pre-treated with the vehicle for 2 days. Thus for each goat there was a comparison between the insulin-stimulated fall in blood glucose before and after treatment with the test material.

Results

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis in the absence of 2-mercaptoethanol revealed the expected heterogeneity of the pituitary bGH extracted by the salt precipitation technique of Ellis (1961) (Fig. 1). There was a major component at M_r 22000 and eight minor components ranging from M_r <16000 to 53000. The rebGH contained a similar major component at M_r 22000 and three minor components with M_r >22000, which corresponded to similar bands in pituitary bGH. The rebGH contained no material of M_r <22000. The high-M_r components were almost absent when the same hormone was run in the presence of 2-mercaptoethanol, thereby suggesting they were disulphide aggregates (results not shown).

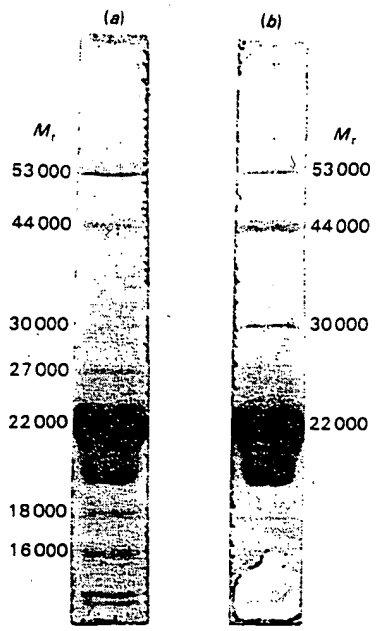

Fig. 1. *Approximate M_r values for (a) pituitary bGH and (b) rebGH obtained by SDS/polyacrylamide-gel electrophoresis*

SDS/polyacrylamide-gel electrophoresis was carried out in 10mM-imidazole/HCl buffer, pH7.0, at 70mA and 0.8V for 4h.

High-pressure liquid chromatography

The results for the high-pressure liquid chromatography are illustrated in Fig. 2 and support the data obtained with SDS/polyacrylamide-gel electrophoresis. The rebGH contained no low-M_r components (i.e. M_r <22000), but contained three high-M_r components (i.e. M_r >22000), two of which corresponded to those found by SDS/polyacrylamide-gel electrophoresis. The pituitary bGH contained at least four peaks of M_r >22000 and a number of low-M_r (i.e. <22000) components.

Radioimmunoassay

When compared on a weight basis, serial dilutions of rebGH were parallel with the pituitary bGH standard (NIH-GH-B2; 1.5 units/mg) in the radioimmunoassay for bovine GH (Fig. 3). The potency of rebGH, derived from these data, was equivalent to 1.6 units/mg. In the radio-

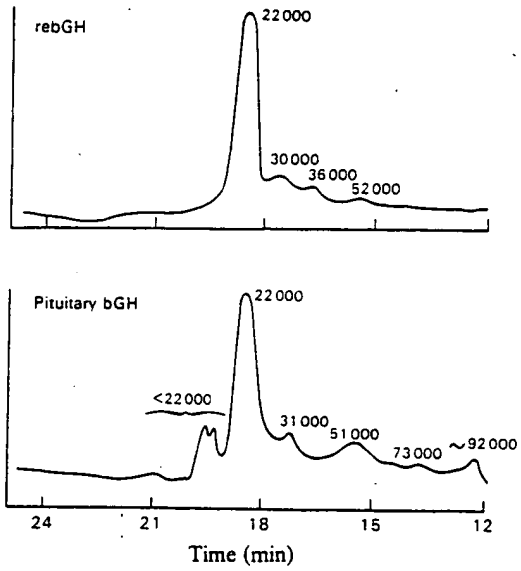

Fig. 2. *Approximate M_r values for rebGH and pituitary bGH obtained by high-pressure liquid chromatography*
High-pressure liquid chromatography was performed with an Ultropac column (TSK 9 3000 SW; 7.5 mm × 600 mm) eluted with 0.1 M-potassium phosphate buffer, pH 7.5, at a flow rate of 0.75 ml/min. M_r values are marked on the Figure.

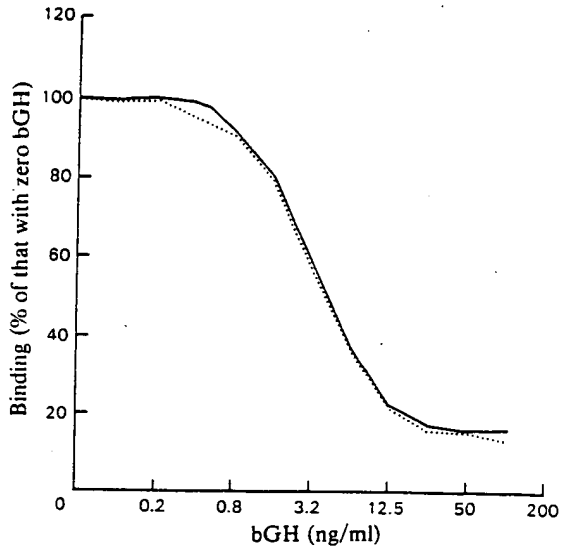

Fig. 3. *Binding displacement characteristics in the radioimmunoassay for ruminant GH (Hart et al., 1975)*
——, pituitary bGH (NIH-GH-B2; 1.5 units/mg); ·····, rebGH.

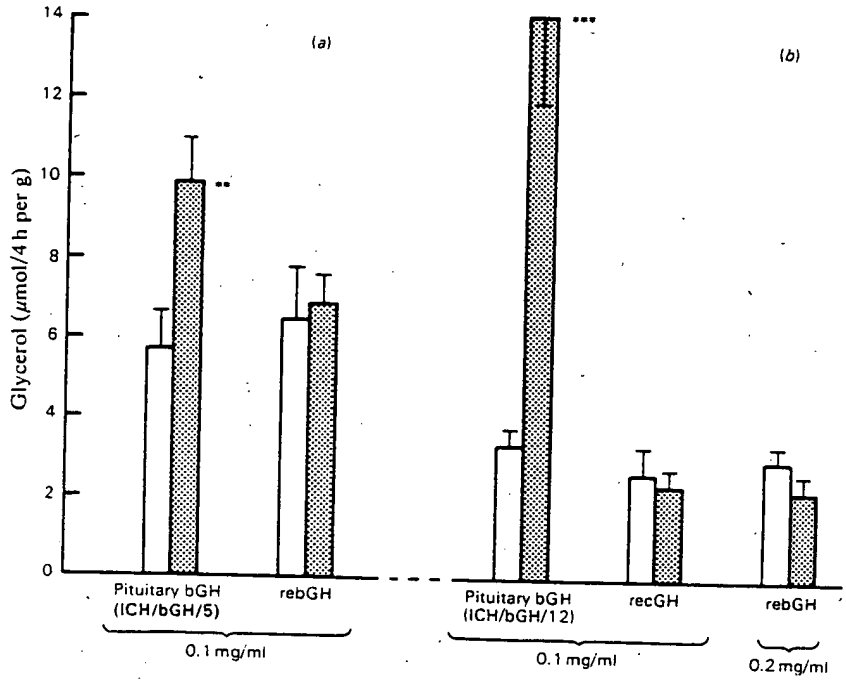

Fig. 4. *Effect of pituitary bGH and bGH and recombinant-DNA-derived chicken GH (recGH) on the release of glycerol from rat epididymal fat maintained in vitro*
Rat epididymal fat was incubated for 4 h (37°C) with either buffer (control; □) or the material to be tested (0.1 or 0.2 mg/ml, ⊡) in the presence of dexamethasone (0.2 μg/ml). Glycerol was analysed by the Boehringer fully enzymic method [$n = 6$ rats/treatment for (*a*) and 5 rats/treatment for (*b*)]. Results are means ± S.E.M.: $^{**}P < 0.01$ and $^{***}P < 0.001$, for comparison of treatment with control, by Student's t test for paired observations.

Table 1. *Growth-promoting activity of recombinant-DNA-derived bovine GH as determined by the hypopituitary-dwarf-mouse assay*

The method of Wallis & Dew (1973) was used for the dwarf-mouse assay. Index of precision (λ) was 0.34 and the 95% fiducial limits of potency were 1.13–2.01. Results are means ± S.E.M. for eight mice.

	Treatment (μg/day)	Weight gain (g)	Potency (units/mg)
Control (saline)		0.12 ± 0.11	
Standard	5	2.05 ± 0.21	
Bovine pituitary GH (NIH-GH-B5; 1.0 unit/mg)	20	2.95 ± 0.32	
	80	3.75 ± 0.19	
Recombinant-DNA-derived bovine GH	10	2.68 ± 0.25	1.4
	40	3.48 ± 0.30	

immunoassay for bovine prolactin, rebGH caused no displacement of ^{125}I-prolactin when tested at concentrations as high as 12.8 μg/ml.

Growth-promoting activity

The results of the hypopituitary-dwarf-mouse assay are given in Table 1 and show that rebGH had a biological activity of 1.4 units/mg.

Lipolytic activity (in vitro)

Fig. 4(*a*) shows the effect of rebGH (0.1 mg/ml) and of pituitary bGH (ICH/bGH/5; 1.2 units/mg; 0.1 mg/ml) on glycerol release from rat epididymal fat maintained *in vitro*. Fig. 4(*b*) illustrates a similar comparison using another preparation of pituitary bGH (ICH/bGH/12; 1.5 units/mg) and recombinant-DNA-derived chicken GH, but the concentration of rebGH was increased to 0.2 mg/ml of medium. Vaughan (1962) had previously established that glycerol is not readily metabolized by rat adipose tissue, and thus its release can be taken as a reliable indication of the lipolytic activity of the material tested. Although both of the pituitary preparations stimulated a significant increase in glycerol production, neither the bovine nor the chicken recombinant-DNA-derived GH was lipolytic *in vitro* at the doses tested.

Intravenous injection into sheep

Single intravenous injection of three preparations of bGH into sheep stimulated similar changes in circulating NEFA (Fig. 5). Pituitary bGH, dissolved in saline at pH 11.0 (group 2), stimulated a significant increase in plasma NEFA after 2 h, which remained elevated for up to 7 h. Similar responses were measured with rebGH (group 3) and pituitary bGH (group 4), which were dissolved in glycine/HCl, pH 3.6, but in both cases the magnitude of the average rise in NEFA was considerably less than that measured in group 2 (group 2, +0.66 mM; group 3, +0.39 mM; group 4, +0.36 mM at 2 h after injection). The initial changes in plasma glucose were different in each group (Fig. 6). For groups 2 and 4, circulating glucose increased within 15 min of injection and then declined to reach a trough after 60–90 min. The sheep treated with rebGH (group 3) showed no initial rise in plasma glucose, but a small fall

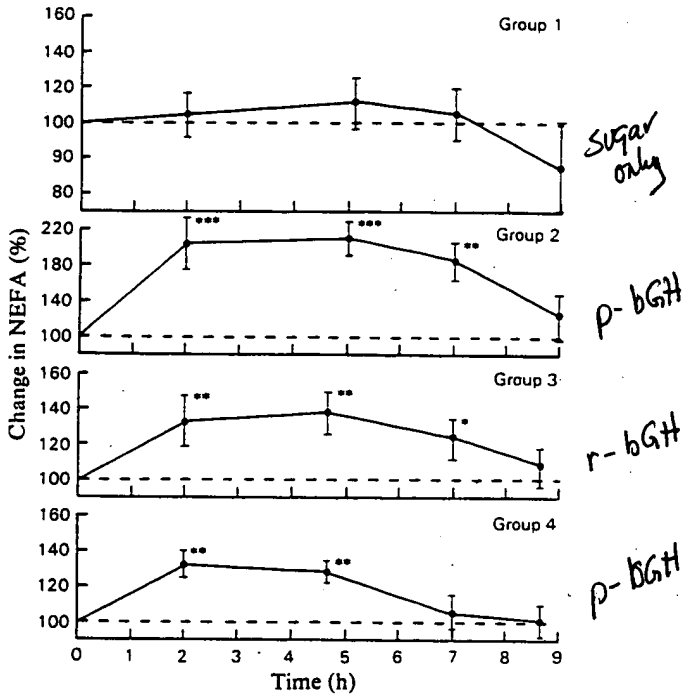

Fig. 5. *Effect of intravenous pituitary bGH and recombinant-DNA-derived bGH on the percentage change in plasma NEFA in sheep*

Four groups each of three sheep were intravenously injected with: group 1, glycine/HCl (0.01 M; pH 3.6) + lactose (0.2%) + mannitol (0.2%); group 2, pituitary bGH (0.15 mg/kg) in sterile saline (0.9%; pH 11.0); group 3, rebGH (0.15 mg/kg) in glycine/HCl (0.01 M; pH 3.6) + lactose (0.2%) + mannitol (0.2%); group 4, pituitary bGH (0.15 mg/kg) dissolved as for group 3. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with the pre-injection value.

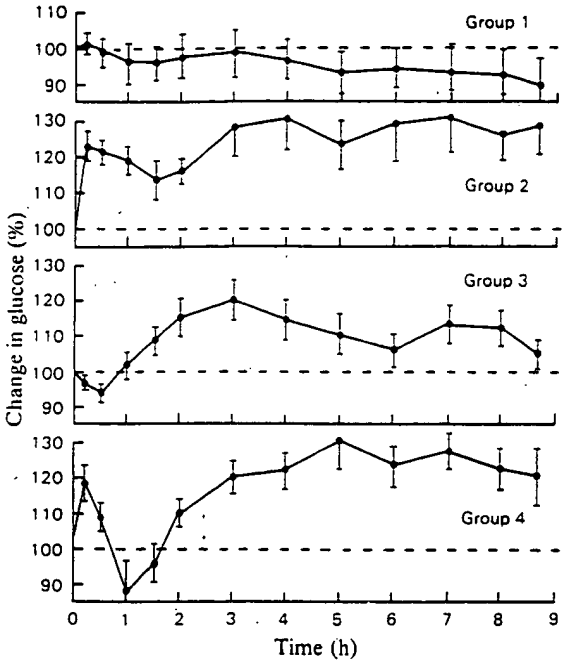

Fig. 6. *Effect of intravenous pituitary bGH and recombinant-DNA-derived bGH on the percentage change in plasma glucose in sheep*
For details, see legend to Fig. 5.

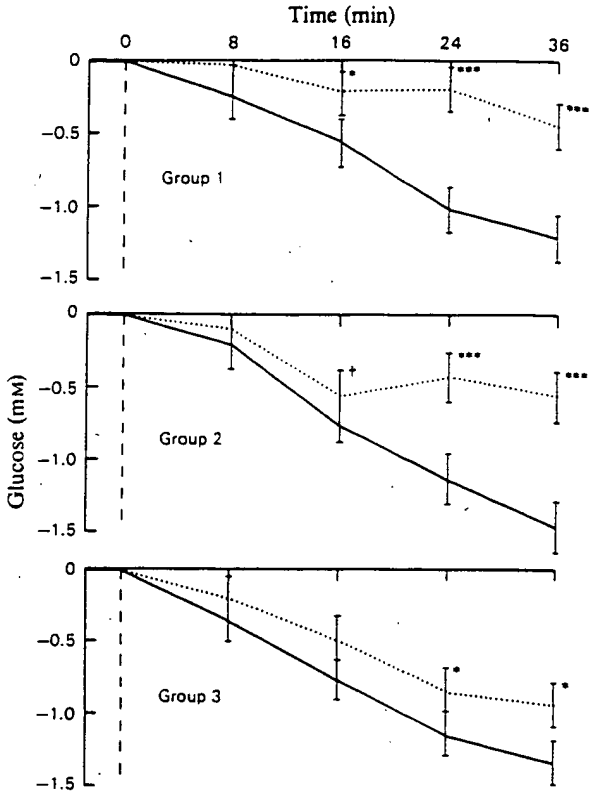

Fig. 7. *Intravenous insulin-tolerance tests in sheep*
Intravenous insulin (0.08 unit/kg)-tolerance tests were conducted in sheep ($n = 4$) before and after subcutaneous injection (0.15 mg/kg, twice daily for 2 days) of: group 1, pituitary bGH in sterile saline (0.9%, pH 11.0); group 2, pituitary bGH in glycine/HCl (0.01 M; pH 3.6) + lactose (0.2%) + mannitol (0.2%); group 3, rebGH dissolved as for group 2. Values are changes in plasma glucose concentrations (±S.E.M.). Statistics refer to a comparison of mean values at each time interval for the experimental treatment (·····) and the control (——): †$P<0.1$, *$P<0.05$, ***$P<0.001$.

was noted 30 min after treatment. Thereafter all of the group exhibited an increase in glucose such that, after 3 h, the average concentration of each group was significantly higher than the average pre-treatment value (group 2, +0.67 mM; group 3, +0.40 mM; group 4, +0.44 mM; $P<0.001$). None of the preparations provoked significant changes in plasma urea.

Diabetogenic activity

Pre-treatment with pituitary bGH dissolved both in saline (pH 11.0) and in glycine/HCl (pH 3.6) significantly decreased the insulin sensitivity of the sheep tested (Fig. 7; groups 1 and 2). The rebGH was also found to be diabetogenic (group 3), but, at the dosage used, its ability to inhibit the insulin-stimulated fall in plasma glucose was somewhat less than that of the pituitary hormone.

Discussion

The data obtained from the radioimmunoassay for ruminant GH clearly indicated that pituitary bGH and rebGH were immunologically indistinguishable with this antiserum. Furthermore, the potency of rebGH, calculated from the radioimmunoassay (1.6 units/mg) was slightly higher than that of the pituitary bGH standard (NIH-GH-B2, 1.5 units/mg), which has the highest biological activity of any bGH preparation distributed under the programmes of the National Institute of Arthritis Metabolism and Digestive Diseases (Reichert & Wilhelmi, 1973, 1978). Allowing for the precision of the dwarf-mouse assay for growth-promoting activity, good agreement was obtained between the potency of rebGH calculated from the radioimmunoassay and that derived from the bioassay (1.4 units/mg). Similar agreement was obtained for recombinant-DNA-derived hGH (Goeddel *et al.*, 1979; Olson *et al.*, 1981). Research in rats has suggested that GH may be modified, both before and after secretion from the pituitary, to a form which either retains or has

enhanced growth-promoting activity but cannot be detected by radioimmunoassay (see Vodian & Nicoll, 1979, for references). The present data indicate that the rebGH used in the present study contains none of these modified forms of GH and, in terms of immunological and growth-promoting activites, is identical with the hormone normally extracted and purified from anterior pituitaries.

The question of whether GH is intrinsically lipolytic or whether this activity is the result of the pituitary hormone being contaminated with either modified forms of GH or other pituitary hormones (e.g. thyrotropin, corticotropin, lipotropin) has occupied researchers for several years (Goodman & Schwartz, 1974; Rao & Ramachandran, 1977). Frigeri (1980) and Frigeri *et al.* (1983) found that highly purified pituitary hGH and bGH failed to stimulate glycerol production from epididymal fat or adipocytes taken from starved rats and that the lipolytic activity *in vitro* was associated with an acidic fraction which was removed during the chromatographic purification. They further demonstrated that recombinant-DNA-derived hGH was not lipolytic when incubated with rat fat *in vitro* (Frigeri *et al.*, 1982).

Our results, with two different recombinant-DNA-derived growth hormones, support those of Frigeri and co-workers. Neither of the preparations increased glycerol production from rat epididymal fat maintained *in vitro*, whereas both of the pituitary preparations were markedly successful in that respect. Although it is possible that rat adipose tissue is insensitive to the lipolytic activity of highly purified bGH, it is more likely that these data support the contention that pituitary bGH, extracted by salt precipitation (Ellis, 1961), contains either modified lipolytic forms of bGH, which may be the result of proteolytic cleavage before or during extraction, or other lipolytic pituitary factors. In this respect it is relevant that the pituitary bGH used in this study contained low-M_r components (i.e. <22000), which were absent from rebGH. Furthermore, it has been shown that commercially available preparations of hGH contain substantial quantities of β- and α-lipotropin (Kuhn *et al.*, 1983).

Intravenous injection of pituitary bGH stimulated a rise in plasma NEFA which was similar to that previously found by others (Williams *et al.*, 1963; Bassett & Wallace, 1966), although the lipolytic activity appeared to be decreased when the hormone was dissolved at the lower pH. The fact that intravenous rebGH increased circulating NEFA in a manner similar to that of the same dose of pituitary hormone suggests two alternatives. Either bGH is directly lipolytic and the molecule is converted into a lipolytic form *in vivo*, or the hormone stimulates/facilitates the activity of a lipolytic intermediate. The former is an attractive possibility, as Pankov *et al.* (1982) have indicated that hGH may have to be proteolytically cleaved before it is able to increase plasma NEFA in rabbits, and evidence has subsequently been obtained to suggest that bGH is modified before it can inhibit the activity of acetyl-CoA carboxylase and thus fatty acid synthesis (Bornstein *et al.*, 1983).

It should be noted that our results and those of Frigeri *et al.* (1982) do not agree with data obtained by Goodman & Grichting (1983) and Goodman (1984), who demonstrated an increase in glycerol production from rat epididymal fat maintained *in vitro* with both highly purified hGH and recombinant-DNA-derived hGH. It is difficult to account for these disparate results; a possible explanation might be that the last group used fed rats in their studies, whereas the other two groups used rats which had been starved for 48 h. Ruiz *et al.* (1981) have shown that the lipolytic response of rat tissue can be influenced by prior feeding, and recombinant-DNA-derived hGH was found to stimulate lipolysis weakly in fat taken from fed rats, but the response was not dose-related (Frigeri *et al.*, 1982).

Similar controversy surrounds the diabetogenic activity of bGH (Lewis *et al.*, 1980). The changes in plasma glucose after intravenous injection of pituitary bGH and rebGH into sheep, combined with the results of the insulin-tolerance test, conclusively indicated that, although the diabetogenic activity of rebGH may be slightly decreased, the hormone can be regarded as intrinsically diabetogenic. This agrees with data in which the diabetogenic activity of recombinant-DNA-derived hGH was examined by using oral glucose-tolerance tests in humans (Rosenfeld *et al.*, 1982). The plasma glucose response to the intravenous injection of pituitary bGH was similar to that found by Bassett & Wallace (1966). It was noteworthy that the increased plasma glucose was associated with the decreased insulin tolerance. In other experiments, in which non-diabetogenic forms of bGH have been examined (Hart *et al.*, 1984), neither of these responses occurred (I. C. Hart, unpublished work), which suggests that the increased circulating glucose is the result of a decrease in the rate of its uptake by body tissues rather than the hormone stimulating glycogenolysis or gluconeogenesis.

The lack of a technique *in vitro* for examining diabetogenic activity prevented us from obtaining further information on the form of the hormone which stimulates this response. However, the conversion of the molecule into a diabetogenic peptide is a likely possibility, as short sequences of hGH are diabetogenic in rats and rabbits (Lostroh

& Krahl, 1976; Bornstein, 1978). Furthermore, Lewis *et al.* (1977, 1980) have increased the diabetogenic activity of purified hGH by proteolytic cleavage with subtilisin and have partially purified a diabetogenic component from crude hGH which has a M_r of less than 5000. The present data do not support the contention that GH can be purified to the point where it is no longer diabetogenic *in vivo*. Preparations of hGH and bGH have been isolated which were growth-promoting but exhibited no diabetogenic activity in three different test systems *in vivo* (Lewis *et al.*, 1977; Hart *et al.*, 1984). Although both preparations had the physical and chemical properties of monomeric GH, the possibility should now be considered that they may have undergone conversion into a non-diabetogenic form of the hormone either before or after extraction from the pituitary.

In conclusion, these results show that the lipolytic and diabetogenic properties of bGH are not the result of contamination with other pituitary factors, but are derived from the bGH molecule. It remains to be seen whether the activities are stimulated by bGH itself, or by proteolytically cleaved forms of the molecule, or whether the hormone activates lipolytic or diabetogenic intermediates *in vivo*.

We are indebted to Dr. D. Fenton for growing the *E. coli* which produced the rebGH and to Mr. A. S. Simmonds for his help with the radioimmunoassays, and also to Miss Michele Cleveland, Miss Lorraine Newman, Miss Victoria Ashton, Mr. A. Jones and Mr. J. Abbey for technical assistance and care of the animals. We are grateful to the National Institute of Arthritis, Metabolism and Digestive Diseases, National Institute of Health, Bethesda, MD, U.S.A., for supplies of bovine prolactin and GH.

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The heterogeneity of bovine growth hormone

Extraction from the pituitary of components with different biological and immunological properties

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Bovine growth hormone (somatotropin) was extracted from anterior pituitaries and fractionated into four protein peaks (A–D) by chromatography on DEAE-Sephacel. Analysis by high-pressure liquid chromatography and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated that the homogeneity of the material increased from fraction A through to D. The properties of the fractions were examined in the following manner: immunological activity (radioimmunoassays for ruminant growth hormone and prolactin); growth-promoting activity (rat tibia test); lipolytic activity (release of glycerol from rat epididymal fat in the presence of dexamethasone); diabetogenic activity (rate of glucose transport in epididymal fat of hypophysectomized rats and intravenous insulin-tolerance tests in goats). None of the fractions contained immunoreactive prolactin and all were equally lipolytic. Although fraction A contained a small quantity of immunoreactive growth hormone it had no growth-promoting or diabetogenic activities. Both fractions B and C were diabetogenic and contained high concentrations of immunoreactive growth hormone, consistent with their growth-promoting activity. Although the growth-promoting activity of fraction D was higher than that of the other three fractions, it was not diabetogenic and was only weakly immunoreactive. These results for bovine growth hormone support the contention that growth hormone, as commonly extracted, is a mixture of different molecular forms and that these different metabolic properties of the hormone might be explained in terms of this heterogeneity.

It has been known for decades that the subcutaneous injection of bovine GH, of variable quality, increases milk yield in cows, goats and sheep (Cowie *et al.*, 1980). However, it is still not known how the hormone exerts its galactopoietic effect. One possibility is that the hormone stimulates lipolysis and/or partitions nutrients away from tissue deposition, thereby increasing the supply of triacylglycerols and glucose to the mammary gland for the synthesis of additional milk (Hart *et al.*, 1978; Hart, 1983). Extensive work with human GH has established that the hormone, as commonly prepared, is not a single molecular entity but a mixture of variants often differing in their biological and immunological activities. By the use of anion-exchange and gel-chromatographic techniques human GH has been separated with neither diabetogenic nor lipolytic activities (Lewis *et al.*, 1980; Frigeri, 1980). Preliminary investigations with the bovine hormone suggested that it might be similarly heterogeneous and that differences in biological activity might be expected (Manchester & Wallis, 1963; Swislocki *et al.*, 1971; Assa & Laron, 1977). None of these studies included an examination of the immunological and diabetogenic activities of the hormone.

The present work describes the fractionation of a crude bovine anterior-pituitary extract into preparations that vary in their immunological and biological (growth-promoting, lipolytic, diabetogenic) properties, with a view to eventually testing the effects of these materials on aspects of intermediary metabolism and milk yield in ruminants.

Abbreviation used: GH, growth hormone (somatotropin).

Materials and methods

Extraction and fractionation

The extraction and fractionation of the pituitaries was based on the method of Wallis & Dixon

(1966) for the chromatographic preparation of bovine GH.

Approx. 100g of frozen bovine anterior pituitaries was partially thawed, homogenized with 300ml of buffer (0.25M-NaCl/6.5mM-$Na_2B_4O_7$/5.5mM-HCl buffer, pH8.7) and stirred for 3h in an ice bath. The extract was centrifuged (10000*g* for 30min at 4°C) and the supernatant retained. The residue was ground with sand, re-extracted for a further 1h in 200ml of the same buffer and centrifuged as before. The combined supernatants were then centrifuged (15000*g* for 30min at 4°C), dialysed against six changes of borate/HCl buffer (6.5mM-$Na_2B_4O_7$/5.5mM-HCl buffer, pH8.7) and frozen overnight.

After centrifugation (30000*g* for 30min at 4°C) the thawed extract was chromatographed on a column (50cm × 19.6cm²) of DEAE-Sephacel (Pharmacia Fine Chemicals), equilibrated with borate/HCl buffer and maintained at 4°C. Elution was continued with this buffer at a flow rate of 110ml/h, and 17ml fractions were collected. Measurements of the absorbance at 280nm showed four protein peaks (fractions A–D; Fig. 1). The contents of the tubes within these peaks were combined, dialysed against three changes of distilled water and freeze-dried.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The method was based on the procedure of Shapiro *et al.* (1967) as modified by Weber & Osborn (1969), and used a 10mM-imidazole/HCl buffer, pH7.0, instead of the phosphate system, to shorten the separation time.

Electrophoresis was carried out on a horizontal thin-layer gel (10% acrylamide) in an LKB 2117 Multiphor apparatus with controlled cooling (5°C) at a constant current of 70mA and a voltage of 0.8V for 4h. Approx. 20μg of protein was applied to each well, and cross-linked haemoglobin (M_r 16000–64000; Sigma Chemical Co.) was run as a standard. Gels were stained for 15h with 0.1% Coomassie Blue R250.

High-pressure liquid chromatography

High-pressure liquid chromatography was performed with an Altex 100A solvent-delivery system coupled to a Pye PV4020 ultraviolet detector. Separation was carried out on an Ultrapac column (TSK G 3000 SW; 7.5mm × 600mm; LKB Instruments), which was eluted with 0.1M-potassium phosphate buffer, pH7.5, at a flow rate of 0.75ml/min. M_r calibration was achieved with a Dalton Mk. VI protein solution (Sigma Chemical Co.). Material (75μg) was dissolved in dilute NaOH, pH9.6 (300μl), and 20μl samples were used for each analysis with detection at 210nm.

Radioimmunoassay

The immunological activity of the material was assessed by using radioimmunoassays specific for bovine prolactin (Hart, 1972) or GH (Hart *et al.*, 1975), with modifications described by Tindal *et al.* (1982).

Growth-promoting activity

Growth-promoting activity was measured by the rat tibia test (Greenspan *et al.*, 1949) as modified by Li (1977), and employed bovine GH (NIH-GH-B15; 0.69 units/mg) as the standard preparation. Male Sprague–Dawley rats were obtained from Olac (Bicester, Oxon, U.K.) and hypophysectomized by the sterotaxic transauricular route (Gay, 1967) at approx. 120g body wt.

Lipolytic activity

The lipolytic activity was evaluated *in vitro* by using epididymal fat-pads from Sprague–Dawley rats (150–180g). The rats were starved for 48h before being killed by cervical dislocation. Both rat pads (approx. 250mg each) were removed, rinsed in 0.9% NaCl at 37°C, blotted and weighed and each was placed in separate siliconized conical flasks (25ml) containing 1ml of KRB buffer (Krebs–Ringer bicarbonate buffer pH7.4; Paul, 1965) containing bovine serum albumin (fraction V, fatty acid-free; Sigma Chemical Co.) (40mg/ml) and dexamethasone (0.22μg/ml). The material to be tested was dissolved in KRB buffer containing bovine serum albumin, and the addition of 0.1ml to the experimental flask gave a final concentration of 0.1mg/ml of medium. The other flask was used as a control (0.1ml, KRB buffer containing bovine serum albumin), and both flasks were incubated for 4h in a shaking water bath (37°C) in an atmosphere of O_2/CO_2 (19:1). Samples of medium for glycerol analysis were removed from all flasks both before and after incubation (Boehringer, fully enzymic method). Each test was replicated five times, and significance was determined by the Student's *t*-test for paired observations.

Diabetogenic activity

In the context of this study diabetogenic activity is defined as the ability of the test material (*a*) to decrease the rate of glucose transport in adipose tissue taken from hypophysectomized rats and (*b*) to impair insulin-sensitivity in goats as measured by the insulin-tolerance test.

(*a*) The effect of the material on glucose transport was measured by using a method similar to that of Zapf *et al.* (1981), with the rate of efflux of non-metabolizable glucose from adipose tissue being used as an indication of changes in glucose

transport (Clausen, 1969). Male Sprague–Dawley rats (body wt. 100g) were hypophysectomized and used only if their body weight did not increase by more than 7g in 3 weeks. Each rat received 0.9% NaCl (control) or the test material dissolved in 0.9% NaCl (100μg/0.2ml per day, subcutaneously) for 3 days before being killed by cervical dislocation. Both epididymal fat-pads (approx. 150mg) were incubated for 70min at 37°C in a siliconized scintillation vial (20ml) containing 3ml of KRB buffer containing bovine serum albumin (15mg/ml), pH7.4, and 30mM-3-*O*-[U-^{14}C]methylglucose (6μCi; Amersham International). After preincubation the pads were rinsed over gauze (8ml of KRB buffer containing bovine serum albumin at 37°C), blotted and re-immersed in 3ml of fresh KRB buffer containing bovine serum albumin and unlabelled 30mM-3-*O*-methylglucose (Sigma Chemical Co.). After 10min the pads were separated from the medium, rinsed and blotted as before, and transferred to another 3ml of KRB buffer containing bovine serum albumin and 3-*O*-methylglucose. Samples (0.1ml) of incubation medium were taken from the vials at 5, 10, 20, 30 and 40min intervals, pipetted into 5ml of Instagel (Packard Instrument Co.) and their radioactivities counted in a liquid-scintillation counter. Immediately after the 40min sample had been taken, the fat-pads were separated from the medium, rinsed with 20ml of 0.9% NaCl at 37°C, blotted and homogenized in 1ml of 1M-NaOH. The fat was digested by heating for 30min in a boiling-water bath, cooled in ice, neutralized with 1ml of 1M-HCl and centrifuged (2000*g* for 30min at 4°C). A sample (0.5ml) of infranatant was removed with a syringe, added to 5ml of Instagel and its radioactivity counted.

All values were expressed as the ratio of the radioactivities present in the incubation medium at time t (c.p.m.$_t$) to the total radioactivity (c.p.m.$_{total}$) present in the medium and tissue at the end of the incubation. This ratio was subtracted from unity (1.0) such that at every time interval this difference reflects the fraction of the total radioactivity released into the medium.

(*b*) Intravenous insulin-tolerance tests were carried out in castrated male goats (body wt. approx. 40kg) that had been pretreated with the test material dissolved in 0.9% NaCl (0.15mg/kg injected subcutaneously twice daily for 3 days). The goats, which had previously been fed *ad libitum*, were starved for 19h before each insulin-tolerance test. On the morning of the test three or four blood samples (5ml) were taken at 10–15min intervals from a jugular catheter, inserted at least 24h previously and the stability of the pre-insulin glucose values was immediately established with a HypoCount blood glucose monitor. Bovine insulin (0.08 unit/kg in 2ml of sterile 0.9% NaCl) was then injected via the catheter followed by 2ml of 0.9% NaCl. Further blood samples were taken at 10min intervals for 30min after the insulin injection, and the plasma was analysed for glucose with a Chemlab continuous-flow analyser. Each test of experimental material was preceded by a control insulin-tolerance test in which the goats had been injected for 3 days with sterile 0.9% NaCl.

Results

Extraction and fractionation

Fig. 1 shows the elution pattern obtained after fractionation of the anterior-pituitary extract on DEAE-Sephacel. On occasions the separation of peaks C and D was less distinct, but they were still readily discernible.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the presence of 2-mercaptoethanol revealed a single component in five preparations of fraction D and an additional band at M_r 44000 in two further preparations. There were three components in fractions C and D, eight components in fraction A and four components in a crude preparation of bovine GH (bGH5) that was prepared by the method of Ellis (1961) and included for comparison. The estimated M_r values of these components are shown in Fig. 2. The

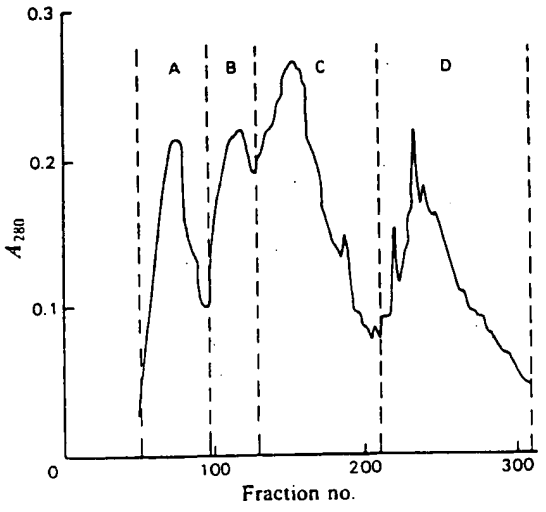

Fig. 1. *Elution profile of bovine anterior-pituitary extract on DEAE-Sephacel*

The column (50cm × 19.6cm^2; 4°C) was eluted with 6.5mM-$Na_2B_4O_7$/5.5mM-HCl buffer, pH8.7, at a flow rate of 110ml/h, 17ml fractions being collected.

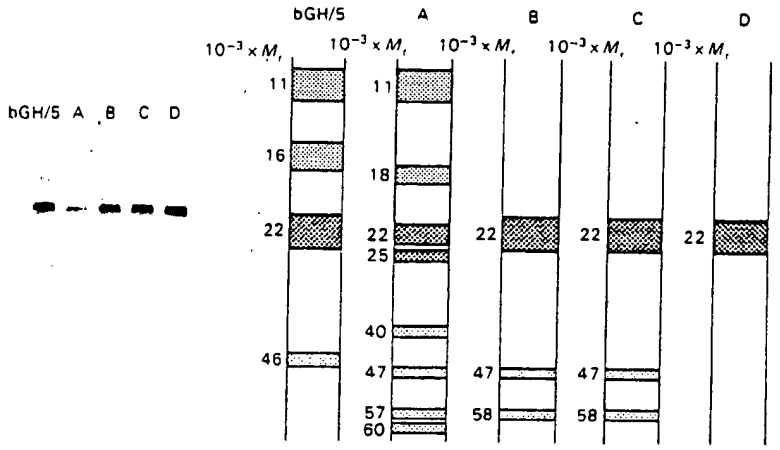

Fig. 2. *Approximate M_r values obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis*
Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out in 10mM-imidazole/HCl buffer, pH 7.0, at 70mA and 0.8V for 4h. A diagram of the electrophoretograms is shown as well as the photograph on which it is based.

22000-M_r band was present to a greater or lesser extent in all of the fractions.

High-pressure liquid chromatography

The results of the high-pressure liquid-chromatographic analysis are illustrated in Fig. 3; the lowest M_r capable of resolution with this system is 20000. Although high-pressure liquid chromatography and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed similar degrees of heterogeneity for the four fractions, there was disparity between the M_r values of the individual components. Fraction A was the most heterogeneous, having several components with M_r values ranging from less than 20000 to 94000. As with the sodium dodecyl sulphate/polyacrylamide-gel-electrophoresis data, fractions B and C appeared to be identical, with four components at M_r <20000, 22000, 38000 and 96000. Fraction D exhibited only one peak, at M_r 22000. It is worth noting that the two preparations of fraction D that exhibited an additional band with sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were found with high-pressure liquid chromatography to contain four components with M_r values ranging from 100000 to <20000.

Radioimmunoassay

Serial dilutions of fractions A, B, C and D were parallel with the bovine GH standard (NIH-GH-B2; 1.5 units/mg) in a specific radioimmunoassay (Fig. 4). The assay shown is less sensitive than usual, as the pre-incubation step was omitted. None of the peaks contained immunoreactive prolactin when tested in the radioimmunoassay at concentrations up to 12.8μg/ml.

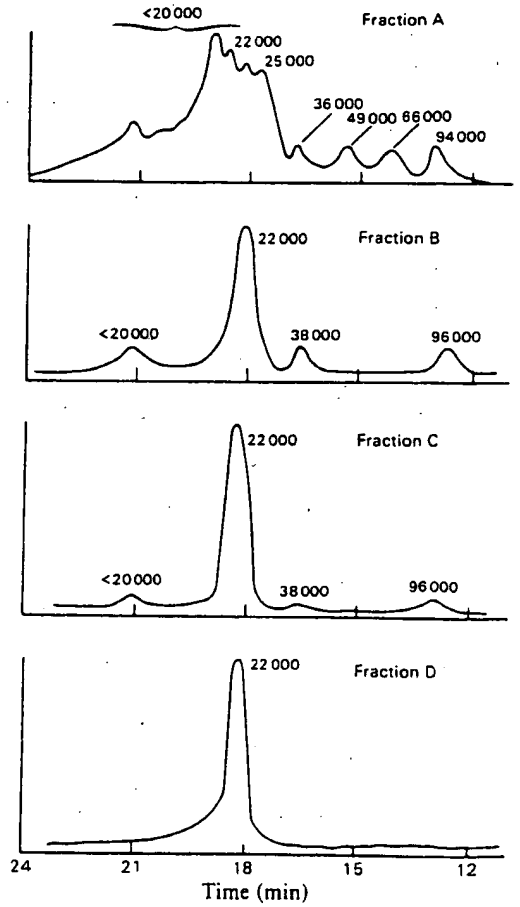

Fig. 3. *Approximate M_r values obtained by high-pressure liquid chromatography*
High-pressure liquid-chromatographic separation was achieved with an Ultropac column (TSK 9 3000 SW; 7.5mm x 600mm) and eluted with 0.1M-potassium phosphate buffer pH 7.5, at a flow rate of 0.75ml/min. M_r values are marked on the Figure.

Growth-promoting activity

Growth-promoting activity tended to increase from fractions A to D (Table 1). The activity of fraction A was low, and the variation between measurements precluded an accurate determination of potency. The relative potencies of the fractions, calculated from the radioimmunoassay inhibition curves (Fig. 4), are given in Table 1 for comparison. Although there was agreement between the bioassay and the radioimmunoassay values for fractions B and C, there was clearly a discrepancy between the high potency value obtained by the rat tibia test for fraction D and the low value measured by radioimmunoassay.

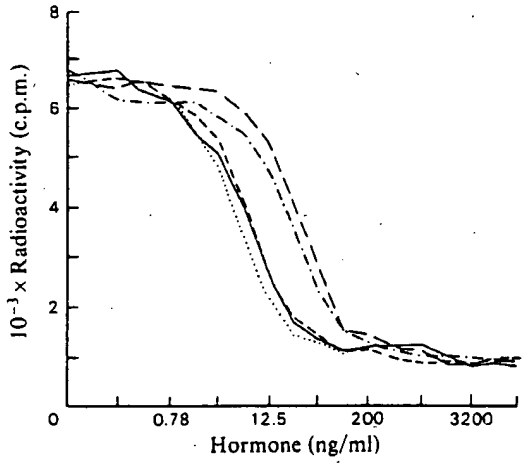

Fig. 4. *Binding-displacement characteristics in the radioimmunoassay for ruminant GH (Hart et al., 1975)* ·····, Bovine GH (NIH-GH-B2; 1.5 units/mg); ——, fraction A; – – – –, fraction B; ——, fraction C; –·–·–, fraction D.

Lipolytic activity

Table 2 lists the lipolytic activity of fractions A–D compared with adrenaline (2 μM) and with bovine GH (NIH-GH-B15; 0.69 units/mg) prepared by a salt precipitation technique. Since glycerol is not readily metabolized by rat adipose tissue, its release can be taken as a reliable indication of the lipolytic activity of the material tested (Vaughan, 1962). All the preparations significantly increased glycerol production in the presence of dexamethasone over a 4 h period. The lipolytic activities of fractions A–D were of the same order as that of bovine GH, but all were less active than adrenaline at the dosage tested. There was a tendency for the lipolytic activity to increase from fraction A to fraction D, but the additional glycerol produced by fraction D was not significantly greater than that stimulated by fraction A.

Diabetogenic activity

(*a*) *Glucose transport.* Fig. 5 demonstrates that there was no significant difference in basal glucose transport in the epididymal fat of hypophysectomized rats pretreated with saline or fractions A and D. However, subcutaneous injection of bovine GH (NIH-GH-B18) and fractions B and C lowered the rate of 3-*O*-methylglucose efflux as compared with the controls. At each time

Table 1. *Growth-promoting and immunological activities of fractions A–D as measured by the rat tibia test and radioimmunoassay*

Radioimmunoassay values were measured against bovine GH (NIH-GH-B2; 1.5 units/mg; Fig. 2). The method of Greenspan *et al.* (1949) as modified by Li (1977) was employed for the rat tibia test. Index of precision for the rat tibia test was 0.46.

	Dose (μg/4 days)	No. of rats	Width of tibial cartilage (μm)*	Potency (units/mg)† Rat tibia test	Radioimmunoassay
Bovine GH (NIH-GH-B15)	Control	10	76 ± 3.4		
	20	10	114 ± 4.3		
	80	12	181 ± 4.9		
	320	9	269 ± 5.2		
Fraction A	40	8	82 ± 7.6	Low	0.25 (0.19–0.35)
	160	7	114 ± 11.4		
Fraction B	40	8	156 ± 4.7	0.86 (0.59–1.31)	0.97 (0.81–1.28)
	160	8	255 ± 5.6		
Fraction C	20	7	140 ± 4.3	1.29 (0.94–1.85)	1.00 (0.84–1.33)
	80	8	210 ± 6.1		
Fraction D	20	7	150 ± 5.1	1.51 (1.01–2.01)	0.29 (0.24–0.37)
	80	7	222 ± 4.0		

* Means ± S.E.M.
† Numbers in parentheses refer to 95% fiducial limits of potency.

Table 2. *Effect of bovine GH, adrenaline and fractions A–D on the release of glycerol from rat epididymal fat in vitro*
Rat epididymal fat was incubated for 4h (37°C) with either buffer (control) or the material to be tested in the presence of dexamethasone (0.2 μg/ml). Glycerol was analysed by the Boehringer fully enzymic method ($n = 5$ rats/treatment).

Fraction	Concn. in medium	Glycerol release (μmol/4h per g)* Control	Treatment	Increase above control (%)	P†
Adrenaline	2 μM	3.4±0.52	9.7±0.40	185.3	<0.001
Bovine GH (NIH-GH-B15)	0.1 mg/ml	4.6±0.35	8.7±0.70	89.1	<0.01
Fraction A	0.1 mg/ml	5.4±0.18	8.9±1.11	64.8	<0.01
Fraction B	0.1 mg/ml	4.6±0.70	8.5±0.81	84.7	<0.01
Fraction C	0.1 mg/ml	4.7±0.62	9.4±0.61	100.0	<0.001
Fraction D	0.1 mg/ml	3.5±0.48	7.3±0.52	108.5	<0.01

* Means±S.E.M.
† Comparison of treatment with control.

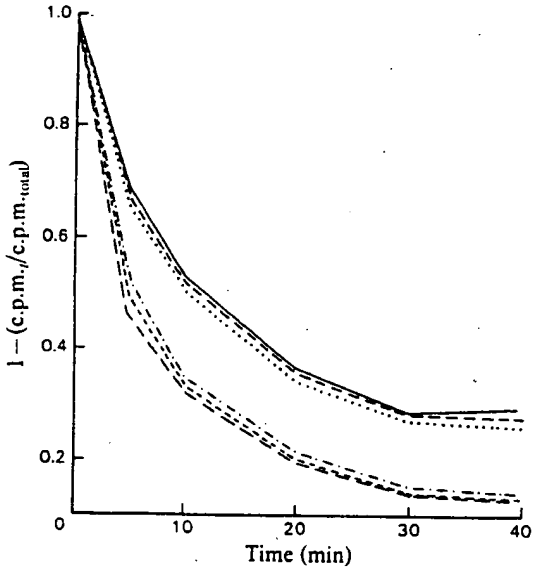

Fig. 5. *Glucose transport in fat from hypophysectomized rats*
Hypophysectomized male rats were pretreated for 3 days (100 μg/0.2ml per day) with bovine GH (NIH-GH-B18; 0.81 unit/mg; ·····), fraction A (——), fraction B (– – – –), fraction C (——), fraction D (–·–·–) or 0.9% NaCl (------) ($n = 5$). The epididymal fat was loaded with 3-*O*-[U-^{14}C]methylglucose and the rate of efflux was measured throughout a 40min period.

interval the efflux for each of these treatments was significantly less than that of the controls at P levels of <0.05 by the paired t-test.

(*b*) *Insulin-tolerance test.* Preparations that lowered the rate of glucose transport in adipose tissue from hypophysectomized rats also caused a marked decrease in insulin-sensitivity at the dosages tested (Fig. 6). Pretreatment with bovine GH and fractions B and C significantly diminished the fall in plasma glucose concentration that occurred after insulin injection in the saline-treated controls. When the goats were treated with fractions A and D, the post-insulin fall in glucose concentration was similar to that found after the control period.

Discussion

The present results lend further support to the concept, based largely on work with the human hormone, that GH, as commonly extracted, is a mixture of different molecular forms and that the different properties of these forms might explain the multiple metabolic activities of the hormone (Lewis *et al.*, 1980).

The A_{280} profiles resulting from chromatography of the bovine anterior-pituitary extracts were similar to those obtained by Wallis & Dixon (1966). However, the use of DEAE-Sephacel instead of DEAE-cellulose clearly improved the separation and made it easier to distinguish the four protein peaks.

The results from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and high-pressure liquid chromatography agreed in some respects but not in others. Both techniques indicated that fraction A was markedly heterogeneous in comparison with the other three fractions and that fractions B and C contained proteins of similar M_r values. Although the 22000-M_r component was present in all of the fractions, the methods did not agree with respect to the M_r values of the other components in each of the four preparations. We have no explanation for these differences beyond the possibility that the same proteins migrated at different rates in the two systems or that the different conditions of analysis caused some of the proteins to dissociate or aggregate. It seems likely that the

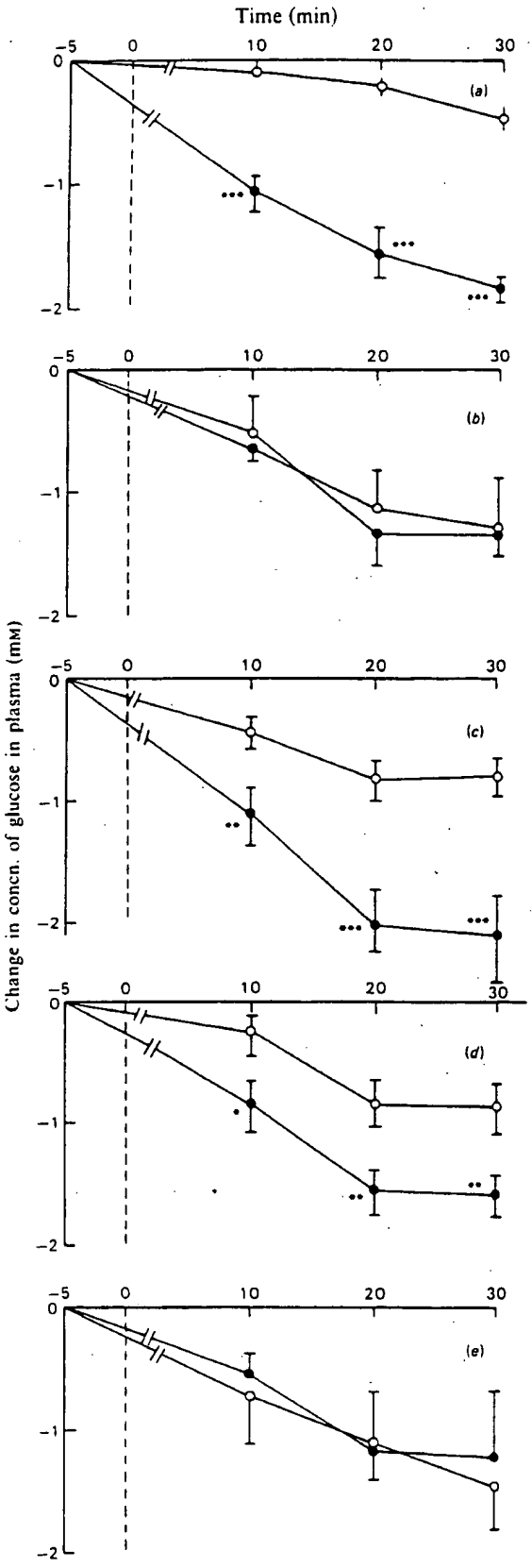

Fig. 6. *Intravenous insulin-tolerance tests in goats*
Tolerance tests towards intravenous insulin (0.08 unit/kg) were conducted in goats both before and after subcutaneous injection (0.15 mg/kg, twice daily) of (*a*) bovine GH (NIH-GH-B18; 0.81 unit/mg), (*b*) fraction A, (*c*) fraction B, (*d*) fraction C and (*e*) fraction D ($n = 3$). Values are changes in plasma glucose concentrations (±S.E.M.). Statistics refer to a comparison of mean values at each time interval for the experimental treatment (○) and control (●). Statistical significance of difference: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

additional components found in two preparations of fraction D were due to incomplete chromatographic separation of fraction D from the other material. More-limited studies with the use of conventional polyacrylamide-gel electrophoresis and polyacrylamide-gel isoelectric focusing also supported the conclusion that fraction D was the most homogeneous (I. C. Hart & S. James, unpublished work).

Parallel inhibition curves were obtained in the radioimmunoassay for ruminant GH and thereby indicated that all of the fractions contained immunoreactive bovine GH, and, when compared on a weight basis, fractions B and C contained the most-immunoreactive material. The absence of immunoreactive prolactin is significant both in terms of the diabetogenic activity of the material (see below) and because most preparations of sheep and bovine GH obtained by salt precipitation contain 1–5% immunoreactive prolactin (Hart, 1972; I. C. Hart, unpublished work). Thus use of this chromatographic procedure for extracting bovine GH yields products that are free of this major hormonal contaminant.

Results from the rat tibia test agreed with those obtained by measuring the weight gain of hypophysectomized rats (Wallis & Dixon, 1966), in that both bioassays detected a tendency for growth-promoting activity to increase from fractions A through to D. Others have used crude bovine GH to prepare fractions that differ in growth-promoting activity (Manchester & Wallis, 1963; Dellacha & Sonenberg, 1964).

The discrepancy between the potency values calculated for fraction D from the rat tibia test and the radioimmunoassay was consistent for all preparations examined, and was of interest as this was the most homogeneous material. Experiments in rats have shown that there was good agreement between bioassay and radioimmunoassay estimates of GH potency when the pituitaries were obtained during a resting state of hormone secretion, but this relationship disappeared when the hormone was measured in pituitaries stimulated to secrete GH (Daughaday *et al.*, 1968; Müller *et al.*, 1970). Similar discrepancies between bioassayable and radioimmunoassayable GH have been found

by others, and have prompted the suggestion that, after suitable stimulation, rat GH may be modified, before or after secretion, to a form that either retains or has enhanced growth-promoting activity but is not identified by antibodies raised against the purified pituitary hormone (Singh *et al.*, 1974; Vodian & Nicoll, 1976, 1977; Ellis *et al.*, 1978). The divergent results obtained by radioimmunoassay and bioassay for fraction D suggest that a similar situation might exist for the bovine hormone.

Extraction and fractionation of pituitary extracts on DEAE-Sephacel failed to produce bovine GH that lacked lipolytic activity. The activity of each fraction was comparable with that of bovine GH produced by the method of Ellis (1961). It is unlikely that the lipolytic activity of the fractions was due to contamination with thyrotropin (Rao & Ramachandran, 1977), as recent administration of these materials to goats (10–20mg, subcutaneously and intravenously) did not raise the plasma triiodothyronine concentration over a 36h period (I. C. Hart, unpublished work). Furthermore, the extraction and fractionation of sheep and human GH by a similar technique yielded a product containing negligible quantities of other anterior-pituitary hormones (Wallace & Ferguson, 1963; Lumley-Jones *et al.*, 1979).

Evidence is accumulating that suggests that the so-called intrinsic lipolytic activity of GH may be due to contamination with either a small-M_r protein or a cleaved form of GH (Salaman, 1972; Assa & Laron, 1977; Schleyer & Voigt, 1977; Frigeri, 1980; Frigeri *et al.*, 1982). Although the markedly heterogeneous fraction A possessed both low growth-promoting and radioimmunoassay activity, its lipolytic activity was similar to that of crude bovine GH and the other fractions. If it is assumed that growth-promoting activity is a property of the pure monomer, this finding tends to support the argument that the lipolytic activity is not an intrinsic property of the molecule.

Measurement of glucose transport by the method of Zapf *et al.* (1981) afforded a rapid evaluation of diabetogenic activity, which was confirmed by the insulin-tolerance test in goats, a species that possesses a form of GH immunologically similar to that of cattle (Hart *et al.*, 1975). The inhibitory influence of bovine GH (NIH-GH-B18) on glucose transport substantiates the work of others, and reinforces the contention that the semi-purified hormone exerts its diabetogenic influence by antagonizing the stimulatory effect of insulin, possibly at a post-receptor site (Schoenle *et al.*, 1981, 1982; MacGorman *et al.*, 1981).

The potency of fraction D in the tibia test in the absence of diabetogenic activity supports the theory that the latter is a property of either a modified form or fragment of GH or some other contaminant. Raben & Westermeyer (1952) were among the first to suggest that pig GH could be prepared without diabetogenic activity, and more recently Lewis and co-workers have discovered that more-homogeneous preparations of human GH are not diabetogenic. They have partially purified a diabetogenic component from crude human GH and claim that this has an M_r value of less than 5000 (Lewis *et al.*, 1977, 1980; Singh *et al.*, 1982). Furthermore, they were able to convert non-diabetogenic human GH into a diabetogenic form by proteolytic modification, thereby suggesting that the active material is a modified form of GH, a contention supported by the fact that short sequences of human GH have been found to be diabetogenic in rats and rabbits (Lostroh & Krahl, 1976; Bornstein, 1978).

However, the diabetogenic activity of the bovine anterior pituitary is not necessarily the exclusive property of GH-related compounds. In 1966 a polypeptide was extracted and found to be diabetogenic in man and dogs (Louis *et al.*, 1966). This material was named bovine diabetogenic peptide and possessed neither growth-promoting activity (Tutwiler & Louis, 1971) nor lactogenic activity (Louis & Conn, 1968). However, evidence is gradually accumulating to suggest this material is closely related to prolactin. The lack of lactogenic activity has been disputed (Tutwiler, 1976), and the physical and chemical properties of bovine diabetogenic peptide and prolactin were very similar (Rogol *et al.*, 1978). Furthermore, we have examined a highly purified preparation of bovine diabetogenic peptide (kindly supplied by Professor L. H. Louis) in the prolactin radioimmunoassay and found a complete cross-reaction with an immunological potency similar to that of the bovine prolactin standard (NIH-P-B2; I. C. Hart, unpublished work). Whatever the conclusions to be drawn from this, the complete absence of immunological prolactin in fractions A–D suggests that none of these materials contained bovine diabetogenic peptide.

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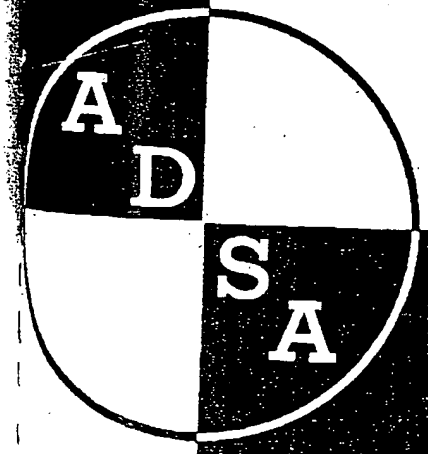

JOURNAL of DAIRY SCIENCE

Responses of High-Producing Dairy Cows to Long-Term Treatment with Pituitary Somatotropin and Recombinant Somatotropin[1,2]

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ABSTRACT

Thirty Holstein cows capable of high milk production were utilized to examine the effects of long-term administration of bovine somatotropin on lactational performance. Treatments were 0 (control), 13.5, 27, and 40.5 mg/day of recombinantly-derived bovine somatotropin and 27 mg/day of pituitary-derived bovine somatotropin. Hormone was injected intramuscularly, once daily beginning at 84 ± 10 days postpartum and continuing for 188 days. Cows were fed ad libitum a total mixed diet throughout the lactation. Recombinant somatotropin treatments increased average fat-corrected milk yield in a dose-dependent fashion from 23 to 41% over the control production (27.9 kg/day. Pituitary somatotropin (27 mg/day) increased milk production by 16%. Milk lactose, protein, and fat composition was similar for all treatments. Control cows were in positive energy balance throughout the treatment period (4.7 Mcal net energy/day. Initially, the large increase in milk yield with somatotropin treatment caused cows to decrease in energy balance. However, voluntary intake gradually increased, and by week 10 of treatment all somatotropin treatment groups were in positive energy balance. Thus, the gain in body weight over the treatment period was similar for all groups, ranging from 17 to 22%. Gross lactational efficiency (milk per unit of net energy intake) was improved by exogenous somatotropin whether calculated as observed, corrected for body weight changes, or using the theoretical energy requirements for maintenance and milk production. Results are consistent with bovine somatotropin as a homeorhetic control that coordinates an array of physiological processes so that nutrients are partitioned for milk synthesis.

INTRODUCTION

The effects of somatotropin (STH; growth hormone) in lactating animals have been the subject of scientific interest for over 50 years. In 1937, Asimov and Krouze (2) first demonstrated that injections of crude pituitary extracts increased milk production in dairy cows. This finding led to an elegant series of studies by Folley, Young, and colleagues aimed at determining whether large-scale utilization of hormone preparations could lead to a significant increase in food production in Britain during World War II [review, (30)]. Although increases in milk production were observed in treated dairy cows, it was concluded that the available quantity of anterior-pituitary tissue (obtained from slaughtered animals) was insufficient to allow for a substantial increase in the nation's milk supply.

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[1]Supported by Cornell University Agricultural Experiment Station and Monsanto Co.
[2]The authors gratefully acknowledge the assistance of staff at Cornell University (M. Partridge, H. N. Erb, G. Birdsall, R. D. Smith, D. M. Galton, W. R. Butler, B. Palhof, B. M. Perkins, T. Kimmich, and the workers at the Cornell University Dairy Teaching and Research Unit) and staff at Monsanto Co. (G. L. Bachman, L. A. Bentle, J. A. Duque, K. A. Dwyer, R. W. Hammond, R. L. Hintz, R. G. Hoffman, K. Jacobson, V. K. Meserole, J. W. Mitchell, F. W. Morgan, J. P. O'Donovan, G. J. Rogan, R. L. Ryan, R. H. Taylor, and D. H. Will).

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Limitations in the supply of STH and in purification techniques have resulted in relatively slow progress in exploring the mechanisms by which STH alters milk production and the potential for commercial application. Studies utilizing highly purified bovine somatotropin (isolated from pituitary glands) have demonstrated that daily injections of exogenous hormone increase milk yield from 10 to 40% [review, (7)]. These studies have generally been short (a few days or weeks), and the pattern of response is a progressive increase in milk yield over the first 4 to 6 days of treatment. When treatment is terminated, milk yields gradually decline to pretreatment levels over a similar interval. Only three studies have involved longer term treatments. Brumby and Hancock (9) conducted a 12-wk study with three sets of identical twins. Milk yield was increased 5.9 kg/day over controls (13 kg/day) but their STH preparation contained some thyrotropic hormone. Cows were fed grain according to milk production and pasture ad libitum. Treatment had no affect on body weight. Using a more highly purified preparation of bovine STH Machlin (15) reported similar increases in milk yield using 3 cows treated for a 10-wk period. In a recent study, Peel et al. (23) utilized five pairs of identical twins and observed a 17.7% (3.5 kg/day increase in milk production for STH-treated cows (22-wk period). There have been no long-term studies with synthetically produced STH, but we have recently demonstrated that recombinantly-derived methionyl bovine somatotropin (MBS) gave increases in milk yield similar to pituitary-derived bovine somatotropin (PBS) in a 6-day study (5). Therefore, our objectives were: 1) to determine the effects of long-term administration of STH on lactational performance and health with special emphasis on mechanism of action, 2) to compare the effects of different doses of MBS, and 3) to compare the effects of MBS and PBS.

METHODS

Thirty multiparous Holstein cows (second to fifth lactation) were assigned to treatments in a randomized block design. Treatments were 0, 13.5, 27, and 40.5 mg/day of MBS and 27 mg/day of PBS. Methionyl bovine somatotropin was produced in *Escherichia coli* via a recombinant plasmid (25). Using conventional techniques, MBS was extracted and purified from the organism to greater than 90% homogeneity with no detectable carbohydrate or deoxyribonucleic acid impurities. Purified MBS was extensively characterized by several biochemical and physical techniques and shown to be structurally and biochemically equivalent to pituitary derived STH with the exception of an additional methionine at the N-terminus (L. Bentle, personal communication). The MBS and PBS were formulated as sterile lyophilized powders with sodium bicarbonate as excipent. Both MBS and PBS were nonpyrogenic as determined by the USP rabbit pyrogen assay. The biopotency of PBS[3] and MBS[3] were similar, approximately 1.4 IU/mg protein (coefficient of variation = 14%), as determined in a hypophysectomized rat weight-gain bioassay. This assay was modified from that reported by Marx et al. (16) and conducted as a slope-ratio assay in which the regressions of body weight gains to log doses of hormone were compared.

Treatments were initiated at 84 ± 10 days postpartum and continued for 188 days. Daily intramuscular injections were administered at approximately 0700 h in one of four alternating sites (right or left gluteal or thigh muscle). Hormone was solubilized in .05 *M* bicarbonate buffer as previously described (20) at a concentration of 9 mg/ml. Thus, daily injection volumes were 1.5, 3.0, or 4.5 ml for the three MBS treatments and 3.0 ml for the PBS and control treatments. If solubilized hormone was not used immediately, it was stored (48 h maximum) at 5°C.

All cows received daily injections of excipient (solubilization vehicle) for 2 wk prior to treatment to allow adjustment to the regimen. Injection volumes were identical to those used during the treatment period. After only a few days, cows adapted to the injection routine and thereafter exhibited no nervousness, discomfort, or evidence of local inflammatory response.

[3]Pituitary bovine somatotropin originated with A. F. Parlow, Department of Obstetrics and Gynecology, Harbor-UCLA Medical Center, Torrance, CA, and both somatotropin sources were obtained from Monsanto Co.

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$b(X_k) + e_{ijk}$, where μ = overall mean; B_i = block effect; T_j = treatment effect; $b(X_k)$ = covariate adjustment for each cow's individual response during the excipient period; and e_{ijk} = residual. Body weight and variables used in gross efficiency calculations were not covariately adjusted. Shape of the lactation curve during the treatment period was evaluated by analysis of polynomial coefficients according to the procedure of Allen et al. (1).

The entire study, including the handling of milk, was conducted in accord with local, state, and federal laws and regulations pertaining to such studies.

RESULTS

The temporal pattern of milk production during the pretreatment and treatment periods is shown in Figure 1. Prior to treatment the lactation curve is characteristic of high-producing cows (with no differences between cows eventually assigned to different treatments). Peak milk production during the pretreatment period occurred at approximately 49 days postpartum (4 wk prior to treatment). During the treatment period, milk yield from control cows decreased in normal fashion, averaging 6 to 7% decline per month. In contrast, STH treatment significantly altered the shape of the lactation curves ($P<.01$). Cows receiving the two higher doses of MBS increased daily production to substantially greater than the peak production observed in early lactation (pretreatment). In fact, their daily milk production remained greater than the pretreatment peak for over 100 days after treatments commenced. Although milk production from cows treated with 13.5 mg/day of MBS did not sharply increase, production did remain relatively constant for several weeks after treatment began. Treatment with PBS caused an initial sharp increase in milk production but thereafter yield declined more rapidly than observed for cows receiving MBS.

During the pretreatment period, average daily production of 3.5% fat-corrected milk (FCM) was similar across groups and averaged 35.9 ± 1.3 kg/day (mean ± SD). Daily injection of MBS resulted in a substantial increase in average daily milk yields over the 188-day treatment period (Table 2). Increases ranged from 6.5 to 11.5 kg/day depending on dose level with the average daily milk yield for the two highest doses being greater during the treatment period than observed in the first 84 days postpartum (pretreatment period). Pituitary bovine somatotropin was less effective, eliciting an increase in milk yield of 4.6 kg/day (+16.5%) over levels observed for controls ($P<.10$). Milk composition was not affected by STH treatment ($P>.10$) (Table 2). Thus, increases in yield of milk fat, protein and lactose paralleled the increases in milk yield observed with somatotropin treatment.

The temporal pattern of net energy intake during the study is shown in Figure 2. During the pretreatment period, cows showed the typical increase in energy intake following the onset of lactation with daily dry matter con-

TABLE 2. Effect of exogenous somatotropin on yield and composition of milk.

Variable[1]	Control	Pituitary bovine somatotropin 27.0 mg/day	Methionyl bovine somatotropin 13.5 mg/day	Methionyl bovine somatotropin 27.0 mg/day	Methionyl bovine somatotropin 40.5 mg/day	SE
Cows, n	6	6	6	6	6	
FCM, kg/day[2]	27.9[a]	32.5[ab]	34.4[bc]	38.0[c]	39.4[c]	1.8
Milk fat, %	3.6	3.3	3.8	3.6	3.6	.1
Milk protein, %	3.4	3.4	3.4	3.4	3.4	.1
Milk lactose, %	4.8	4.8	4.9	4.8	4.9	.1

[a,b,c]Means in same row with different superscripts differ ($P<.05$).

[1]Treatment period was 188 days commencing at 84 ± 10 days postpartum. Response data (weekly means) were adjusted by covariance analysis using each individual cow's response during the excipient period.

[2]FCM = 3.5% Fat-corrected milk.

Cows were selected from the Cornell University herd and had acclimated to the facilities during previous lactations. Breeding was initiated after 60 days postpartum. After parturition cows were moved to a tie stall barn and housed in this unit throughout the study. The unit had automatic ventilation and artificial lighting. The lighting was continuous and varied from approximately 258 lx (14 h/day) to 54 lx (10 h/day) as measured at shoulder height of the cows. Cows were moved to the parlor twice daily for milking at 0300 and 1500 h. Once weekly, a.m. and p.m. milk samples were obtained for analyses of lactose, fat, and protein. Lactose was determined by an enzymatic analysis according to Eppard et al. (12). Milk fat and protein were analyzed by infrared techniques (3).

Cows were fed ad libitum one of three total mixed diets (Table 1). Orts were recorded and fresh feed offered once daily at 1000 h. Weekly feed samples were composited monthly and analyzed by New York Dairy Herd Improvement Cooperative. The three diets were formulated so that typical intake would support nutrient requirements (19) for 37, 27, and 20 kg/day milk production. To allow full expression of potential for milk production, shifts to the lower energy density diets were relatively conservative. All cows were fed the high energy diet from parturition through the first portion of the treatment period. Thereafter, when milk production (weekly average) decreased to 25 and 17 kg/day, cows were switched to the medium and low energy diets, respectively.

Body weights were determined on two consecutive days every other week. Weighings were conducted as cows returned from the p.m. milking. Net energy (NE) balance was calculated on a weekly basis. Milk energy was calculated using average milk yield and percent fat according to Tyrrell and Reid (28). Net energy for maintenance was according to National Research Council (NRC) (19) using a 4-wk rolling average for body weight.

Statistical analysis was conducted using Statistical Analysis System (26) for the randomized block model, $Y_{ijk} = \mu + B_i + T_j +$ $b(X_k) + e_{ijk}$, where Y_{ijk} = observation; μ = overall mean; B_i = block effect; T_j = treatment effect; b = regression coefficient; X_k = covariate adjustment; e_{ijk} = response during the treatment period; e_{ijk} = residual. Body weight, milk yield, and gross efficiency calculated. Shape of the treatment period of polynomial coef procedure of Allen et

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TABLE 1. Composition of the complete mixed diets fed through the course of lactation.

Ingredients and nutrients[1]	Diet		
	High energy	Medium energy	Low energy
Ingredient[2]			
Corn meal, %	41.8	30.2	14.0
Hay crop silage, %	20.0	29.3	38.3
Corn silage, %	19.6	25.2	37.5
Soybean meal, %	15.3	12.3	8.5
Nutrient content			
Net energy, lactation, Mcal/kg	1.63	1.56	1.52
Crude protein, %	17.2	15.8	16.1
Acid detergent fiber, %	17.4	21.9	25.7
Calcium, %	.821	.855	.766
Phosphorus, %	.44	.41	.40
Magnesium, %	.30	.34	.34
Potassium, %	.97	1.16	1.48
Sodium, %	.315	.405	.204
Sulfur, %	.31	.32	.26
Iron, ppm	246	353	291
Zinc, ppm	59	58	54
Copper, ppm	9	8	10
Manganese, ppm	42	56	58

[1] All values presented on dry matter basis.

[2] In addition, sodium bicarbonate (.8%) was included in the high and medium energy formulations and all formulations contained mineral supplements added as needed to meet National Research Council recommendations (19).

TABLE 2. Effect of ex

Variable[1]	Con
Cows, n	6
FCM, kg/day[2]	27.5
Milk fat, %	3.(
Milk protein, %	3.(
Milk lactose, %	4.1

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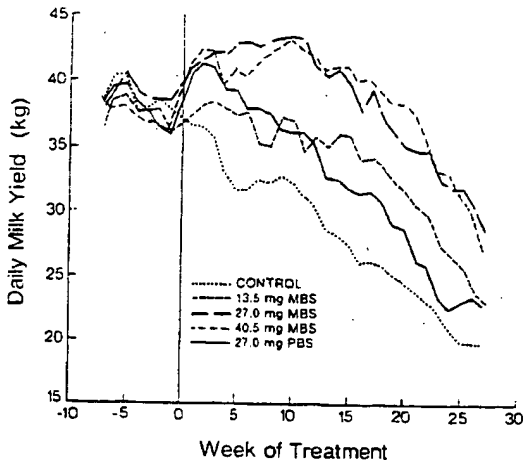

Figure 1. Effect of exogenous bovine somatotropin on milk yield. Treatments commenced at week 0 (84 ± 10 days postpartum) and continued for 27 wk. Milk production data (unadjusted) represent weekly averages. Daily dose of methionyl bovine somatotropin (MBS) and pituitary bovine somatotropin (PBS) as indicated.

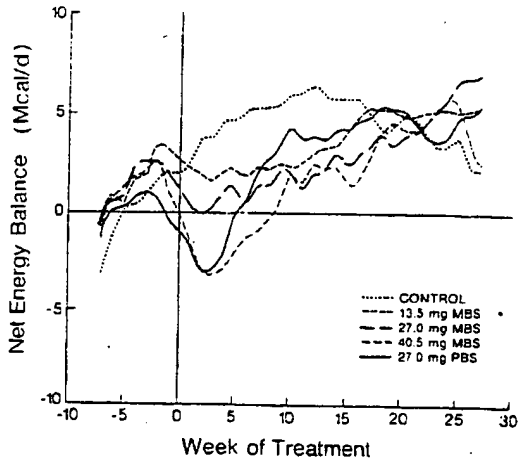

Figure 3. Effect of exogenous bovine somatotropin on net energy balance (Mcal/day). Treatment period as in Figure 1. Net energy balances (unadjusted) represent 3-wk rolling averages. Daily dose of methionyl bovine somatotropin (MBS) and pituitary bovine somatotropin (PBS) as indicated. d = Day.

sumption in the last 2 wk pretreatment averaging 3.9 ± .4 (mean ± SD) percent of body weight. Net energy intake remained similar for the first 5 wk of the treatment period. However, there was a trend for an increase in net energy intake by most STH treatment groups, especially those receiving the 27.0 and 40.5 mg/day of MBS (Figure 2). At weeks 9 to 11, daily dry matter intake for the cows receiving the two highest doses of recombinant-STH averaged 4.6 ± .3% of body weight as compared with 4.0 ± .5% for the controls ($P<.05$). Greater differences in net energy intake between treatment groups are apparent for the last half of the treatment period, but these primarily represent animals on some treatments shifted to lower energy diets in response to decreased milk production. The first shifts from the high energy to the

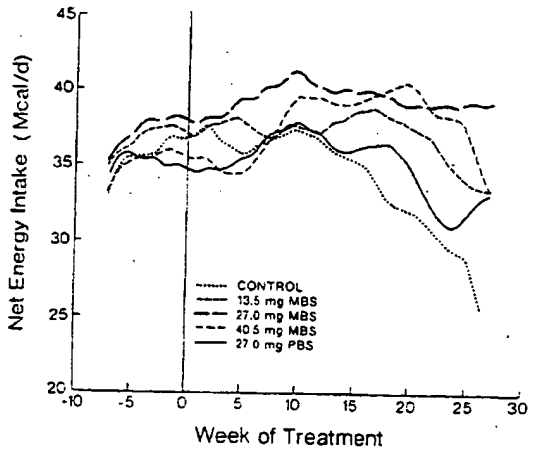

Figure 2. Effect of exogenous bovine somatotropin on net energy intake (Mcal/day). Treatment period as in Figure 1. Net energy intakes (unadjusted) represent 3-wk rolling averages. Daily dose of methionyl ovine somatotropin (MBS) and pituitary bovine somatotropin (PBS) as indicated. d = Day.

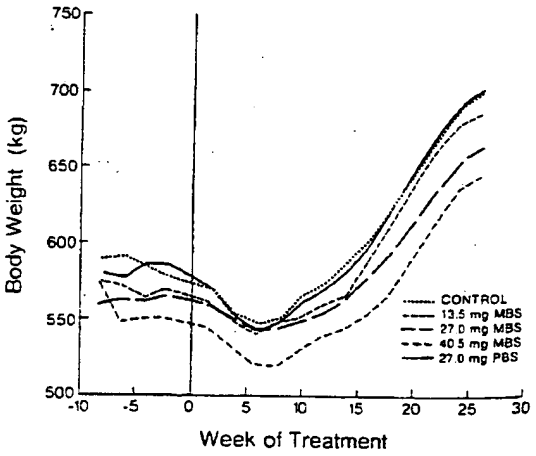

Figure 4. Effect of exogenous bovine somatotropin on body weight. Treatment period as in Figure 1. Body weights (unadjusted) represent 4-wk rolling averages. Daily dose of methionyl bovine somatotropin (MBS) and pituitary bovine somatotropin (PBS) as indicated.

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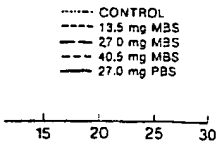

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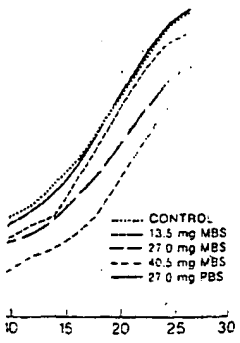

Treatment

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medium energy diet occurred during the 12th and 13th wk of treatment (individual cows in the PBS and control groups). Overall, the shift from the high energy diet to the medium energy diet occurred at weeks 20 ± 10 (mean ± SD), 24 ± 7, 25 ± 4, 28 ± 1 and 29 ± 4 for the control; PBS; and low, medium, and high MBS treatment groups, respectively. Thus, the changes in energy intake early in the treatment period were not confounded by diet. In addition, milk yield from animals receiving the two high MBS treatments was great enough that cows essentially remained on the high energy diet throughout the entire treatment period. These group differences are reflected in the average net energy intake for the total treatment period (Table 3).

The net energy balance through the pretreatment and treatment period is shown in Figure 3. For controls, the net energy balance ranged from +3 to +6 Mcal/day throughout the treatment period. Thus, milk production of the control animals was not limited by energy intake. The net energy balance had a different pattern for the STH treatment groups. Initially, the increase in milk yield caused cows to decrease in energy balance and, for those treatments which had substantial initial increases in milk yield (PBS and the two higher doses of MBS), some cows were in negative energy balance. However, by the 10th wk of treatment, the increase in feed intake resulted in all STH treatment groups being in positive energy balance. The average energy balance across the treatment period was positive for all STH groups (Table 3) and sufficient to replenish body stores used in early lactation (Figure 3, pretreatment period).

The changes in body weight across the treatment period (Figure 4) are consistent with the energy balance data. Over the 188-day interval, treatment groups had similar body weight gains ($P>.10$) ranging from 124 kg (+22%) for the control group to 92 kg (+17%) gain for the cows receiving 40.5 mg/day of MBS (Table 3).

Expressing gross lactational efficiency as kg FCM/Mcal NE intake showed a trend for somatotropin treatment to increase this ratio (Table 3). A dose-dependent pattern was evident for cows receiving MBS, but only the 27 and 40.5 mg treatments resulted in a significant increase over the control value ($P<.05$). However, the observed gross efficiency does not account for changes in body weight. Assuming increments of body weight gain had equal net energy across treatments (18), similar differences between treatments were apparent when the gross efficiency was corrected for tissue gains (Table 3). Theoretical gross efficiency was also calculated using NRC (19) requirements for maintenance and milk production. Theoretical and corrected gross efficiency values were relatively similar within a treatment (Table 3). This has implications on the mechanism of action for STH. Similarity between corrected and theoretical gross efficiency could occur if STH treatment alters nutrient partitioning but has no effect on the digestibility of dietary energy or on the net energy requirements for maintenance, milk, and tissue gain.

The dramatic increases in milk yield sustained over the 188-day treatment period obviously indicate that STH did not stress the animals adversely. Animals were in good health throughout the study. Nine cows were pregnant prior to treatment, 19 cows conceived during the treatment period, and 2 cows (one control, one 40.5 mg MBS/day) did not conceive. All health and reproduction parameters (including clinical observations, physical examinations, somatic cell counts, conception rate, services per conception, and gestation length) were at or better than resident herd averages.

DISCUSSION

This study clearly demonstrates that the long-term administration of bovine somatotropin causes remarkable increases in milk production of dairy cows. As shown in Figure 5, the response to recombinant STH is dose-dependent, ranging from 23.3 to 41.2% increase in milk yield over the dose range (13.5 to 40.5 mg/day). These responses are particularly impressive because cows were excellent producers even before treatment. Using the pretreatment production, the projected (29) 305-day milk yield was over 9600 kg. The shape of the dose response curve with MBS (Figure 5) is similar to that reported by Eppard et al. (12) in a short-term study with pituitary-derived bovine somatotropin. However, further studies are required to determine if the dose titration results for MBS are consistent across a variety of different conditions.

TABLE 3. Effect of exogenous somatotropin on energy utilization and body weight during the treatment period.[1]

Variable[1]	Control	Pituitary bovine somatotropin 27.0 mg/day	Methionyl bovine somatotropin 13.5 mg/day	Methionyl bovine somatotropin 27.0 mg/day	Methionyl bovine somatotropin 40.5 mg/day	SE
Net energy intake, Mcal/day	34.1	35.1	36.7	39.2	37.5	1.8
Milk energy secretion, Mcal/day	19.6[a]	22.3[a]	23.3[ab]	26.8[b]	26.6[b]	1.4
Net energy balance, Mcal/day	4.7[a]	2.9[ab]	3.7[ab]	2.8[ab]	1.7[b]	1.0
Body weight[2]						
Prior to treatment, kg	583	584	572	558	559	18
End of treatment, kg	707	709	684	673	651	27
Change during treatment, %	22	21	20	21	17	3
Gross efficiency,[3] kg FCM/Mcal NE intake						
Observed	.83[a]	.90[ab]	.93[abc]	1.00[bc]	1.03[c]	.04
Corrected[4]	.94[a]	1.04[ab]	1.03[ab]	1.10[b]	1.12[b]	.03
Theoretical[5]	.96[a]	1.00[a]	1.03[ab]	1.07[b]	1.07[b]	.02

a,b,c Means in the same row with different superscripts differ ($P<.05$).

[1] Treatment period was 188 days commencing at 84 ± 10 days postpartum. Data are not adjusted for pretreatment response.

[2] Body weights are for 2-wk period immediately before treatment commenced and for a 2-wk period at the end of treatment.

[3] FCM = 3.5% Fat-corrected milk; NE = net energy. All gross efficiency values (observed, corrected, and theoretical) were calculated on an individual cow basis.

[4] Values are corrected for body weight gain on an individual cow basis using a value of 6.0 Mcal NE for each kg of body tissue gain (18). Corrections were made by subtracting the NE for tissue gain from NE intake and then computing gross efficiency using the adjusted NE intake.

[5] Calculated using the actual FCM produced during the treatment period divided by the NE requirement calculated from NRC (19) requirements for maintenance and milk using actual FCM and body weights for cows during the treatment period.

Figure 5. Relatior somatotropin (mg/da Treatment was for 1: days postpartum wit average yields of 3.5' drawn through the r values (•), but the s somatotropin (□) is = Day.

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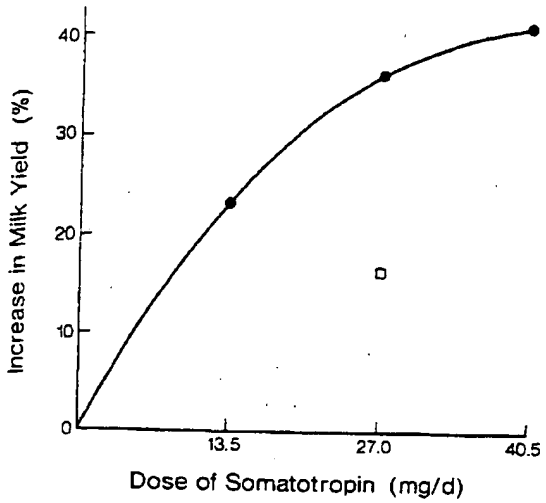

Figure 5. Relationship between dose of bovine somatotropin (mg/day) and milk yield response. Treatment was for 188 days commencing 84 ± 10 days postpartum with percent increases based on average yields of 3.5% fat-corrected milk. Graph is drawn through the methionyl bovine somatotropin values (•), but the single dose of pituitary bovine somatotropin (□) is also depicted for reference. d = Day.

The interval of STH treatment in this study was week 13 to week 39 postpartum. Treatments were terminated after 188 days because of limited PBS supply rather than biological considerations. Treatments were not initiated until 84 ± 10 days postpartum so that the pretreatment performance data could be utilized for a more accurate statistical evaluation of treatment effects given the limited animal numbers. In addition, at the time this study was initiated, literature reports (short-term studies) conflicted as to the efficacy of exogenous STH treatment prior to peak milk production [see review, Bauman and McCutcheon (7)]. However, recent short-term (<2 wk) studies with highly purified PBS have demonstrated a lactational response even in very early lactation (days 20 to 29 and days 41 to 46 postpartum) when cows are in substantial negative energy balance (17, 24).

Lactational response to the daily administration of PBS differed in some regards to that observed with MBS. Average milk production during the treatment period was increased by 16.5 and 36.2% in response to 27.0 mg/day (equimolar) of PBS and MBS, respectively (Table 2). Although similar in potency in a rat growth assay (16), PBS and MBS clearly differ in bovine lactational potency as evidenced by the milk response curves during the treatment period (Figure 1). The biological basis for this difference is not obvious. Blood samples were obtained at regular intervals throughout the study and no significant antibody titers to STH were detected with any of the treatments (data not presented). Overall, the increase in milk yield (+4.6 kg/day) we observed for the pituitary-STH group is similar to earlier reports involving long-term treatment with PBS. Brumby and Hancock (9) observed an increase of 5.9 kg/day (control = 13 kg/day) in a 12-wk study. Machlin (15) presents results from his 10-wk study in graphic form, but increases were approximately 5 kg/day over control yields of 10 to 15 kg/day. However, in both of these studies the shape of the milk response curve during the treatment period was similar to what we observed for the recombinant hormone treatments (i.e., initial increase followed by maintenance at a relatively constant level for the next 10 to 12 wk). In a recent 22-wk study by Peel et al. (23), daily treatment with pituitary somatotropin (50 mg/day; .78 IU/mg) gave increases in milk yield (+3.5 kg/day; + 17.7%), which also were similar to our results.

Results from this study demonstrate that STH is a homeorhetic control involved in the orchestration of many physiological processes for the directed partitioning of nutrients to support the requirements for milk synthesis (4, 6). As pointed out previously (15, 20, 30), this likely involves two major facets. First, STH must alter the metabolism of body tissues so that more nutrients are partitioned to the mammary glands and, second, STH must enhance the ability of the mammary gland to synthesize milk.

The extensive array of physiological processes being altered by STH include the metabolism of carbohydrate, protein, lipid, and minerals as reviewed by Bauman and McCutcheon (7). They emphasize that the milk yield responses to STH are perfectly coordinated with the alteration in the metabolism of body tissues as evidenced by the fact that steady state concentrations of blood metabolites are maintained. Data in the present long-term study are consistent with these observations. Certainly the dramatic increases in milk yield with STH

treatment could not persist through the treatment period (Figure 1) unless coordinated changes occurred in body metabolism so that steady-state conditions were maintained. These changes must have involved the metabolism of lipid, carbohydrate, and amino acids, because milk composition was not altered (Table 2). Short-term studies using PBS also have observed that milk composition is unaltered when cows are in positive energy and nitrogen balance (5, 12, 13, 21, 22). In contrast, the percent milk fat increases and the percent milk protein decreases if lactational response to STH treatment causes cows to be in negative energy and protein balance (12, 20, 22, 24, 27).

A second facet of the response to long-term STH treatment involves the mammary glands. Mammary response could be passive and merely the consequence of an increased supply of nutrients to the gland because of STH's effects on other body tissues. Although this is possible, it seems unlikely. Supplying extra nutrients to cows that are already consuming sufficient nutrients to meet their requirements has not been shown to change the shape of the lactation curve or increase the synthesis of all milk components as in this study (10, 14).

The increase in milk synthesis with STH treatment most likely involves changes in the activity of key regulatory enzymes resulting in an increased synthesis rate per epithelial cell. However, the possibility of an increase in mammary cell numbers cannot be excluded. In either case, somatotropin affects the maintenance of lactation as evidenced by the changes observed in the shape of the lactation curve (Figure 1). This role would presumably involve an altered turnover of epithelial cells and cellular components so that the normal loss of cells or the normal decline in biochemical activity per cell was reduced with STH treatment.

Somatotropin receptors have not been identified in cow mammary tissue. Therefore, the effects on the mammary gland as well as other tissues could be indirect. It has been suggested that STH effects on skeletal muscle and cartilage are indirect and mediated by somatomedins (polypeptide hormones produced by other tissues such as liver in response to STH). Recent studies have reported that circulating somatomedin C are elevated in STH-treated dairy cows (11, 23). Therefore, the effects of STH may be mediated by an array of polypeptide hormones, including somatomedins, which coordinate complex changes in tissue metabolism and provide communication among organs and cells (7).

Of special interest was the effect of long-term treatment with STH on feed intake and energy balance. Our results clearly demonstrate that high-producing cows were able to replenish body reserves adequately during the treatment period in spite of the increases in milk production (Table 3, Figure 3). The diets were formulations typically used for group feeding by dairy farmers. The high milk production of the STH-treated cows did, however, result in their remaining on the highest energy diet much longer than the controls. In addition, STH-treated cows were able to replenish body reserves by increasing voluntary intake. In our study, the voluntary intake tended to increase over the first 10 wk of treatment, particularly in the groups with the greatest milk response (27.0 and 40.5 mg/day treatments). A change in voluntary intake of STH-treated animals was more clearly evident in the 22-wk study by Peel et al. (23). Their animals were pasture fed and by week 8 of somatotropin treatment, the voluntary intake of pasture had increased consistent with the increased milk production. Thus, on a chronic basis, changes in voluntary feed intake are another component of the homeorhetic responses to exogenous STH. However, the chronic regulation of feed intake is most likely associated with rates of tissue metabolism rather than STH per se.

The effects of STH appear to center entirely on nutrient partitioning. Previous short-term studies with PBS have demonstrated that the digestibility of dietary energy, carbon, and nitrogen is not altered by STH treatment (20, 27). In addition, STH treatment (2 wk) had no effect on the maintenance requirement or the partial efficiency of milk synthesis (27). Our results from long-term treatment with STH are consistent with these observations as evidenced by the similarity within treatment between the corrected gross efficiency (kg FCM/Mcal NE consumed, adjusted for tissue gain) and the theoretical gross efficiency calculated using NRC requirements (Table 3). Therefore, if STH changed digestive processes, maintenance re-

quirement, or the partial efficiency of milk synthesis, effects must be subtle and not detectable by present experimental methods.

Bauman et al. (8) examined the physiological basis for animal differences in milk production and concluded that the regulation of nutrient partitioning is the major source of animal variation. They emphasized this regulation would be complex and multifaceted but that improvements in productive efficiency were dependent on developing an understanding of the biological control of nutrient utilization. The present study clearly demonstrates that STH is one key component of this control. It is impressive that even in high-producing cows, manipulation of this single control (by administration of exogenous STH) can increase milk yield by up to 41%. The extra milk produced requires a predictable amount of additional nutrients. Thus, treatment with exogenous STH increases the productive efficiency of dairy cows by dilution of costs associated with maintenance. This, of course, is precisely the mechanism for the increased productive efficiency observed in genetically superior cows and the basis for improvements made through the use of artificial insemination and genetic selection (8).

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Roughage Content
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